# CHONDROCYTE DEATH IN INJURED ARTICULAR CARTILAGE – *IN VITRO* EVALUATION OF CHONDROPROTECTIVE STRATEGIES USING CONFOCAL LASER SCANNING MICROSCOPY

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## DECLARATION

The work presented in this thesis was performed at the Centre for Integrative Physiology, School of Biomedical Sciences, University of Edinburgh. The composition of this thesis is my own work.

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## DEDICATION

To my wife Alison, for always being there for me

To my parents, for educating me

## ABSTRACT

A reproducible *in vitro* model of mechanically injured (scalpel cut) articular cartilage was developed in this work utilising bovine and human osteochondral tissue. Using fluorescence-mode confocal laser scanning microscopy (CLSM), the model allowed (1) spatial and temporal quantification of *in situ* (within the matrix) chondrocyte viability following a full thickness cartilage injury and (2) serial evaluation of three chondroprotective strategies in injured bovine and human articular cartilage: (a) medium osmolarity (b) medium calcium concentration and, (c) subchondral bone attachment to articular cartilage.

Medium osmolarity significantly influenced superficial zone chondrocyte death in injured (scalpel cut) bovine and human articular cartilage. Greatest percentage cell death occurred at 0 mOsm (distilled water). Conversely, a raised medium osmolarity (600 mOsm) was chondroprotective. The majority of *in situ* cell death occurred within 2.5 hours of the experimental injury, with no further increase over 7 days.

Exposure of articular cartilage to calcium-free media significantly decreased superficial zone chondrocyte death in injured (scalpel cut) articular cartilage compared with exposure to calcium-rich media (2-20 mM). In calcium-rich media, the extent of percentage cell death increased with increasing medium calcium concentration but remained localised to the superficial zone of injured articular cartilage over 7 days. However, in calcium-free media, there was an increase in percentage cell death within deeper zones of injured articular cartilage over 7 days.

Excision of subchondral bone from injured (scalpel cut) articular cartilage resulted in an increase in chondrocyte death at 7 days that occurred in the superficial zone of injured as well as the adjacent uninjured regions of articular cartilage. However, the presence of subchondral bone in the culture medium prevented this increase in chondrocyte death within the superficial zone. Subchondral bone may have interacted with articular cartilage via soluble mediator(s) that influenced chondrocyte survival. In human articular cartilage, healthy subchondral bone also interacted with articular cartilage in explant culture and promoted *in situ* chondrocyte survival, while sclerotic subchondral bone was detrimental to chondrocyte viability.

These findings are of translational relevance to fluid management systems used during open and arthroscopic articular surgery, clinical and experimental research into cartilage injury, repair and degeneration as well as current techniques of tissue engineering.

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## ABBREVIATIONS

| | |
|---|---|
| AS | Articular surface |
| BSA | Bovine serum albumin |
| CE | Cut edge |
| CLSM | Confocal laser scanning microscopy |
| CD | Cell density |
| $CD_{FT}$ | Cell density within full thickness of articular cartilage |
| $CD_{SZ}$ | Cell density within superficial zone of articular cartilage |
| CMFDA | 5-chloromethylfluorescein diacetate |
| DZ | Deep zone |
| FCS | Fetal calf serum |
| ICRS | International Cartilage Repair Society |
| MZ | Middle zone |
| OCJ | Osteochondral junction |
| $PCD_{FT}$ | Percentage cell death within full thickness of articular cartilage |
| $PCD_{SZ}$ | Percentage cell death within superficial zone of articular cartilage |
| PI | Propidium iodide |
| ROI | Region of interest |
| SZ | Superficial zone |
| TUNEL | Terminal deoxynucleotidyl transferase dUTP nick end labelling |

# CHAPTER 1

# INTRODUCTION

## 1.1 Articular Cartilage

### 1.1.1 Composition

Articular cartilage, also called hyaline cartilage, is the smooth, glistening white connective tissue that covers the articulating ends of bones within synovial joints. In most individuals, articular cartilage will provide normal joint function over their lifetime. Such resilience of the tissue is remarkable because articular cartilage is avascular, alymphatic and aneural. Moreover, the tissue comprises only a single cell type – the chondrocyte – which synthesises and maintains an extracellular matrix that provides a skeleton for a functional, biological joint surface capable of withstanding movement under load (Buckwalter & Mankin 1998b). Chondrocytes synthesise the macromolecular constituents of the extracellular matrix - collagen, proteoglycans and non-collagenous proteins (Buckwalter *et al.* 1998b;Hunziker, Quinn, & Hauselmann 2002). The extracellular matrix in turn acts as a signal transducer allowing chondrocytes to detect alterations in its macromolecular composition, so that matrix synthesis and degradation remain intricately balanced under the control of chondrocytes (Buckwalter *et al.* 1998b).

Up to 80% of the wet weight of articular cartilage is water (Buckwalter *et al.* 1998b;Stockwell & Meachim 1973). Although there is some variation between species, articular chondrocytes generally comprise only a small proportion (less than 10%) of the total tissue volume (Stockwell & Meachim 1973). Each cell is therefore surrounded by a relatively large volume of extracellular matrix. The two main components of the extracellular matrix are proteoglycans and collagen. Proteoglycans have a half-life of several months, whereas collagen has a half-life

spanning several decades (Goldring 2006). Since the location and appearance of the articular chondrocytes may remain unchanged for such long periods, these cells are often considered to be metabolically inactive. However, individual chondrocytes are surprisingly metabolically active and have glycolytic rates per cell comparable to other cells in vascularised tissue (Buckwalter *et al.* 1998b;Goldring 2006;Stockwell & Meachim 1973) - the metabolic activity appears low because of the low overall cell density within the tissue.

### 1.1.2 Structure

Despite the simplicity of its composition, the structure of articular cartilage is complex and heterogeneous. In mature adult articular cartilage, each chondrocyte is linked at its surface to a transparent pericellular glycocalyx which in turn is enclosed by a fibrillar pericellular capsule (Poole, Flint, & Beaumont 1984;Stockwell & Meachim 1973). This microanatomical unit is called a chondron and single, double or linear chondron columns are visible in articular cartilage even at low power (x10) light microscopy (Poole 1997;Stockwell & Meachim 1973) (**Figure 1.1**).

Individual chondrocyte morphology and extracellular matrix organisation varies according to the depth from the articular surface and it is possible to divide articular cartilage into four layers or zones – superficial, middle, deep and calcified - although the boundaries between each zone cannot always be precisely defined (Poole *et al.* 1984;Poole 1997;Buckwalter *et al.* 1998b;Hunziker *et al.* 2002;Stockwell & Meachim 1973). In the superficial zone, flattened, ellipsoid-shaped chondrocytes lie within a matrix consisting of relatively low proteoglycan content and layers of

collagen fibres orientated parallel to the articular surface (Poole *et al.* 1984;Jeffrey, Gregory, & Aspden 1995;Poole 1997). This arrangement is thought to serve two main functions. First, it provides tensile strength to the tissue allowing it to act as a tension resisting diaphragm (Stockwell & Meachim 1973). Second, it acts simultaneously as a filter for cell nutrients from synovial fluid and as a barrier to tissue damage from the larger molecules of the immune system (Maroudas 1973; Buckwalter *et al.* 1998b;Stockwell & Meachim 1973).

In the middle zone, chondrocytes become more spherical and the proteoglycan content increases (**Figure 1.1**). The collagen fibres arch to align more obliquely to the joint surface (Poole *et al.* 1984;Jeffrey *et al.* 1995;Poole 1997). The deep zone is characterised by rounded chondrocytes that align themselves in columns perpendicular to the articular surface with the highest concentration of proteoglycans (**Figure 1.1**). The collagen fibres are radially arranged and extend into the tidemark, the boundary between uncalcified and calcified cartilage (Poole *et al.* 1984;Jeffrey *et al.* 1995;Poole 1997). Ultrastructural studies using scanning electron microscopy suggest that the tidemark may serve as a tethering mechanism for the relatively flexible and perpendicularly oriented collagen fibrils of the deepest portion of the non-calcified articular cartilage and may prevent them from being sheared at their point of anchorage to the calcified layer (Redler *et al.* 1975). The calcified layer separates the articular cartilage from subchondral bone and is characterised by rounded chondrocytes surrounded by uncalcified lacunae (Poole 1997) or calcified cartilage (Buckwalter *et al.* 1998b) and radial collagen fibres anchored in the calcified matrix (Poole *et al.* 1984).

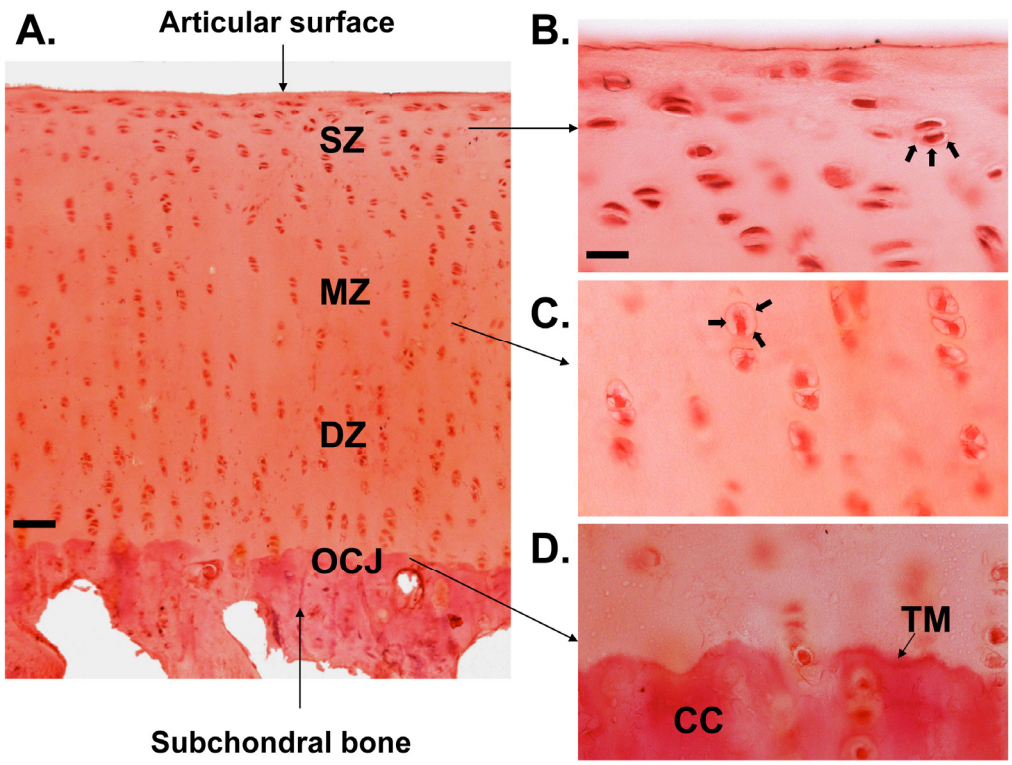


**Figure 1.1: Articular cartilage histology**

A 10μm thick coronal section was cut with a microtome after dehydration and paraffin embedding of a bovine cartilage explant. The section was stained with Mayer's haematoxylin (stains the cytoplasm of chondrocytes in varying shades of pink) and safranin-O (stains proteoglycan orange-red).

**(A)** Low magnification (x10) photomicrograph showing the entire thickness of articular cartilage and the osteochondral junction (OCJ). Note the heterogeneous organisation of the chondrocytes: cells in the superficial zone (SZ) are oval, flat and orientated parallel to the articular surface, whereas those in the middle zone (MZ) and deep zone (DZ) are rounder and arch to orientate more obliquely (MZ) and perpendicular (DZ) to the articular surface (black bar = 80μm).

**(B-D)** Higher magnification (x40) photomicrographs of the SZ, MZ and OCJ of articular cartilage respectively, showing the heterogeneity in chondrocyte morphology in greater detail. Each chondrocyte is enclosed in a chondron (block arrows) which appear as single, double or columnar units. The tidemark (TM) separates the uncalcified and calcified cartilage (CC). There is comparatively less proteoglycan staining (orange-red) in the SZ compared with the MZ and DZ indicating the difference in the proteoglycan content within these zones (black bar = 20μm).

The cell density of articular cartilage (number of chondrocytes per unit volume) also varies with increasing depth from the articular surface. There are difficulties with defining the cell density for each zone as the boundaries between the layers are not distinct entities but represent a gradual transition in cellular morphology and tissue structure. However, qualitative and quantitative histological assessment indicates that the highest cell density in articular cartilage occurs at its articular surface (within the superficial zone) and gradually decreases with increasing distance from the articular surface (Hunziker *et al.* 2002;Stockwell & Meachim 1973). Intriguingly, the precise reason for the greatest cell density immediately below the articular surface is still not known. It has been suggested that this region may act as a metabolic 'sink' (Stockwell & Meachim 1973) that regulates the environment within the deeper regions of articular cartilage and the increased cell density is necessary for the associated high nutritional, metabolic and mechanical demands placed on the superficial zone. Cell density also varies between different joints of the same species and between the same joints in different species (Stockwell & Meachim 1973). In general, there is an inverse relationship between cell density and the size of the joint or indeed, the size of the species. Interestingly, cell density is also inversely related to the thickness of articular cartilage. These observations suggest that the absolute number of cells lying below a unit area of articular cartilage may be relatively constant between species (Stockwell & Meachim 1973). Articular cartilage varies in cell density, matrix composition, thickness and mechanical properties within the same joint, among different joints in the same species and between different species. However, in all synovial joints articular cartilage has the same overall structure and performs the same functions (Stockwell & Meachim 1973).

While the extraordinary functional and load-bearing capabilities of articular cartilage can be attributed to the cell-matrix interactions within the tissue, its longevity relies almost entirely on the highly specialised chondrocyte synthesising, maintaining, organising, repairing and degrading the extracellular matrix (Buckwalter & Mankin 1998a;Buckwalter *et al.* 1998b). As the sole constituent cell of articular cartilage, the chondrocyte is the focus of this work.

## 1.2 Mechanical injury to articular cartilage

Articular cartilage is subjected to mechanical load in all synovial joints. The duration, frequency, magnitude and direction of this load (force) varies within different joints and within different regions of the same joint. Despite its durability, normal articular cartilage can be mechanically injured by a force that exceeds its capacity to maintain tissue homeostasis and structural integrity. A mechanical injury to articular cartilage can be considered in two component parts: (1) the cartilage injury or 'wound' created by the mechanical force and, (2) the cartilage repair response to the mechanical injury.

### 1.2.1 Cartilage injury

In a complex, heterogeneous, anisotropic tissue like articular cartilage, the cartilage injury has temporal (time-dependent) and spatial (depth-dependent) features. The initial mechanical insult to articular cartilage results in chondrocyte death and matrix disruption within minutes-hours (Tew *et al.* 2000;Redman *et al.* 2004;Huntley *et al.* 2005a;Bush *et al.* 2005b). Interestingly, experimentally injured regions of articular cartilage also exhibit a delayed, secondary phase of chondrocyte death that

progresses from the wound edge over a period of several days (Tew *et al.* 2000;Redman *et al.* 2004). While these temporal aspects of the cartilage injury have been well studied after blunt and sharp forms of mechanical trauma (Tew *et al.* 2000;Chen *et al.* 2001;Levin *et al.* 2001;D'Lima *et al.* 2001a;Redman *et al.* 2004), the spatial distribution of chondrocyte death and matrix damage has received less attention. This is partly because the injured cartilage has mainly been visualised using thin coronal sections (Tew *et al.* 2000;Chen *et al.* 2001;Levin *et al.* 2001;D'Lima *et al.* 2001a;Redman *et al.* 2004) which may not appreciate the entire extent of the tissue injury in three-dimensions. For example, the histology section in **Figure 1.1** demonstrates the coronal cut surface of the full thickness of articular cartilage but does not visualise the articular 'surface' unless tangential sections are also obtained. This region of the articular cartilage immediately below its surface may be of particular importance in terms of the mechanical injury as it has the highest cell density and may comprise 30-50% of all cells within the volume of tissue (Wong *et al.* 1996;Hunziker *et al.* 2002;Stockwell & Meachim 1973).

### 1.2.2 Cartilage repair

In contrast to cartilage injury, there is a substantial body of research investigating the repair response of articular cartilage. It has even been suggested that perhaps no experiment has been performed more frequently than mechanically injuring articular cartilage and observing the manner in which it heals over time (Mankin 1982). It is now accepted that in mature, healthy, adult articular cartilage, there is no DNA replication or cell division *in vivo* and chondrocytes may be considered to be post-mitotic (Goldring 2000;Parsch *et al.* 2002;Archer & Francis-West 2003). The

literature also supports the view that articular cartilage has a limited capacity for spontaneous repair due to its avascularity. Partial thickness defects do not heal and full thickness defects repair with structurally and mechanically inferior fibrocartilage (Mankin 1982;Buckwalter *et al.* 1998a;Buckwalter *et al.* 1998b;Hunziker 2002).

Vascular tissues such as skin and viscera heal by the established phased responses of necrosis, inflammation and repair (Mankin 1982). Partial thickness articular cartilage injury (not penetrating the junction of calcified cartilage and subchondral bone) will result in tissue necrosis but the inflammatory and repair responses are absent and hence, these lesions show no healing response (Mankin 1982;Shapiro, Koide, & Glimcher 1993). In full thickness articular cartilage injury (penetrating the junction of calcified cartilage and subchondral bone), a healing response does occur because the vascularised subchondral bone provides a source of blood which forms a clot within the cartilage defect (Hunziker 2002). The blood clot is rich in repair cells (including neutrophils, fibroblasts, macrophages, mesenchymal stem cells), fibrin matrix, growth factors and inflammatory mediators and initiates a healing response (Mankin 1982;Hunziker 2002). However, for reasons that remain to be determined, this healing response is still rudimentary and hyaline tissue is replaced by fibrocartilage which is structurally and mechanically inferior (Mankin 1982;Hunziker 2002). Further, loading of this substandard surface predisposes to joint degeneration with pain and loss of function (Hunziker 2002;Squires *et al.* 2003).

### 1.2.3 Cartilage preservation after cartilage injury

Experimental injury of articular cartilage - for instance, with a scalpel or trephine - is associated with a 'zone' of chondrocyte death and extracellular matrix degradation at the wounded edge (Tew *et al.* 2000;Redman *et al.* 2004). In these novel experiments, blunt wounds were made initially with a trephine in full depth bovine articular cartilage explants. The explants were subsequently cut in half through the centre of the trephine wound with a sharp scalpel to produce blunt and sharp trauma on the same explant. Using terminal deoxynucleotidyl transferase dUTP nick end labelling (TUNEL) with ultrastructural analyses to assess chondrocyte death, $^{3}$H-thymidine labelling to assess cell proliferation and antibodies (COL2-3/4M and BC-13) to detect changes in matrix turnover, the experiments on blunt trauma demonstrated a band of cell death adjacent to the lesion edge with no new matrix synthesis or cell proliferation after 10 days. In contrast, with sharp trauma the cartilage wound demonstrated new matrix synthesis and more limited cell death. The experiments suggested that sharp mechanical injury causes less chondrocyte death compared to blunt impacts and as a consequence, the surviving cells retain the capability to synthesise and repair the damaged extracellular matrix. Similar preservation of *in situ* chondrocyte viability also occurs when human articular cartilage is wounded with a scalpel (sharp trauma) rather than a circular osteotome (blunt trauma) (Huntley *et al.* 2005a). In a further *ex vivo* study, bovine articular cartilage discs (6 mm) were incubated in media containing inhibitors of necrosis (Necrostatin-1) or apoptosis (Z-VAD-FMK) before cutting a 3 mm inner core (Gilbert *et al.* 2009). This core was left *in situ* to create disc/ring composites, cultured for up to six weeks with the inhibitors, and analyzed for cell death, glycosaminoglycan release, and tissue

integration. Treatment of articular cartilage with cell death inhibitors during wound repair increased the number of viable cells at the wound edge, prevented matrix loss, and resulted in a significant improvement in cartilage-cartilage integration (Gilbert *et al.* 2009).

The available evidence indicates that while a mechanical injury to articular cartilage damages the cells and the matrix, the burden of the repair response in injured articular cartilage is incumbent upon the surviving cells. In contrast to the major research efforts that have been directed at understanding mechanisms of cartilage repair, the concept of cartilage preservation at the time of injury has received little attention. The emphasis of this work is on defining the temporal and spatial distribution of the initial chondrocyte death that follows the mechanical injury and developing strategies that may help decrease its extent. Since this phenomenon of cartilage preservation from injury is most relevant to the cellular component of cartilage, it is referred to as ‘chondroprotection’ in the remainder of the work.

### 1.2.4 Clinical relevance of chondroprotection

Intra-articular fractures are common traumatic injuries involving a disruption of the articular surface that usually requires anatomical reduction and rigid fixation (Dirschl *et al.* 2004). There are no reliable estimates of the prevalence of cartilage defects due to athletic injuries, osteochondritis dissecans, osteonecrosis or indeed, localised cartilage degeneration, but open and arthroscopic reconstructions of these defects are becoming increasingly commonplace in orthopaedic practice worldwide (Hunziker 2002). Since cartilage has a poor repair response, reconstructive surgery for injured

articular cartilage aims to alleviate pain, improve function and prevent joint degeneration by restoring a congruous articular surface. A variety of different surgical techniques have been designed to promote intrinsic healing or transplant extrinsic tissue into the cartilage defect (Hunziker 2002). However, such surgery does not predictably prevent joint degeneration or indeed, alleviate pain and loss of function (Hunziker 2002;Dirschl *et al.* 2004). A major limitation to successful articular reconstruction is the 'paradoxical' mechanical injury to healthy cartilage from instruments and implants used during surgical procedures such as osteotomes, motorised shavers, intra-articular pins and screws (Hunziker 2002;Huntley *et al.* 2005a). Such 'surgical injury' results in a zone of chondrocyte death that limits successful lateral integration across the interface between host and repair tissue (Hunziker 2002;Redman *et al.* 2004;Huntley *et al.* 2005a;Gilbert *et al.* 2009). It follows that decreasing the zone of chondrocyte death from the surgical injury may increase the proportion of viable cells at the injured edge. This may promote integrative cartilage repair and as a result, help overcome some of the problems inherent with fibrocartilagenous repair that have limited a multitude of experimental and therapeutic manoeuvres (Hunziker 2002).

### 1.3 Confocal laser scanning microscopy (CLSM)

In an anisotropic tissue like cartilage (Jeffery *et al.* 1991), it would be important to quantify the responses of *in situ* chondrocytes - cells embedded within their native matrix – to define the temporal and spatial distribution of chondrocyte death following mechanical injury. This allows a full appreciation of the intrinsic zonal heterogeneity. Moreover, characterisation of *in situ* cell viability responses to

mechanical injury is more likely to be representative of the *in vivo* situation compared to that of chondrocytes isolated from their native extracellular matrix (Roach, Shearer, & Archer 1989;D'Lima *et al.* 2001d;Kuhn *et al.* 2004). In the past, it has been difficult to visualise *in situ* cellular responses without physically disturbing the native tissue architecture. Advances in microscopic techniques have allowed the visualisation of undisturbed living cells without physical sectioning of the tissue. Confocal laser scanning microscopy (CLSM) is one such type of high resolution fluorescence microscopic method that produces three dimensional images of *in situ* tissue microstructure. This imaging technique is central to the hypotheses investigated in this thesis and a brief summary of its principles is detailed.

### 1.3.1 Overview

Confocal microscopy was pioneered by Minsky over 50 years ago and although current CLSM designs incorporate sophisticated, computer driven systems, the key elements of his original concept have been retained and refined (Minsky M 1988). A confocal microscope creates a sharp image of a specimen that would otherwise appear blurred with a conventional microscope by excluding light from the specimen that is not from the focal plane of the microscope. The image acquired represents a thin cross-section of the specimen. This cross-section is acquired without physical sectioning of the specimen and is thus referred to as an ‘optical section’. A consecutive series of optical sections obtained along the vertical axis of the specimen can be combined using imaging software to build a three-dimensional reconstruction of the imaged volume of the specimen.

### 1.3.2 Principles

The principle of CLSM is based on focussing light on a single point on the specimen and collecting some of the returning rays. This avoids the undesirable scattered light that obscures the image when the entire specimen is illuminated at the same time. In addition, the light returning from the specimen passes through a second pinhole aperture that rejects light rays that are not directly from the focal point and allows collection of only the in-focus light from the focal point (Minsky M 1988). Modern confocal microscopes incorporate this concept of point-by-point illumination and rejection of out-of-focus light by using a high intensity laser light source to image a point on the specimen, dichroic mirrors that reflect light shorter than a certain wavelength but transmit light of longer wavelengths, a pinhole to reject out of focus light and a set of motorised rotating mirrors that allow the laser to scan across the entire specimen (**Figure 1.2**). 'Optical sectioning' occurs when the light passes onto a detector (photomultiplier tube), attached to a computer which builds up the cross-sectional image one pixel at a time. Optical sections imaged at short and regular intervals along the vertical axis of the specimen (by moving the stage on which the specimen is placed) are then combined to create a three-dimensional rendition of the imaged volume of the tissue. For biomedical research, most confocal microscopes acquire optical sections by stimulating fluorescence from fluorophores that are used to bind to specific components of the specimen (so called 'fluorescence-mode' CLSM). Multiple fluorophores may be applied to the specimen making them the only visible parts with CLSM. The laser light source is used to excite the fluorescently labelled regions of the tissue with an excitation wavelength that is close to the peak excitation wavelength of the fluorophore. The fluorophore, excited by the laser

source, emits a longer wavelength that is directed back along the same optical path as the illuminating beam. With point-by-point illumination and rejection of out-of-focus light, optical sections of fluorescently labelled components of the tissue are generated.

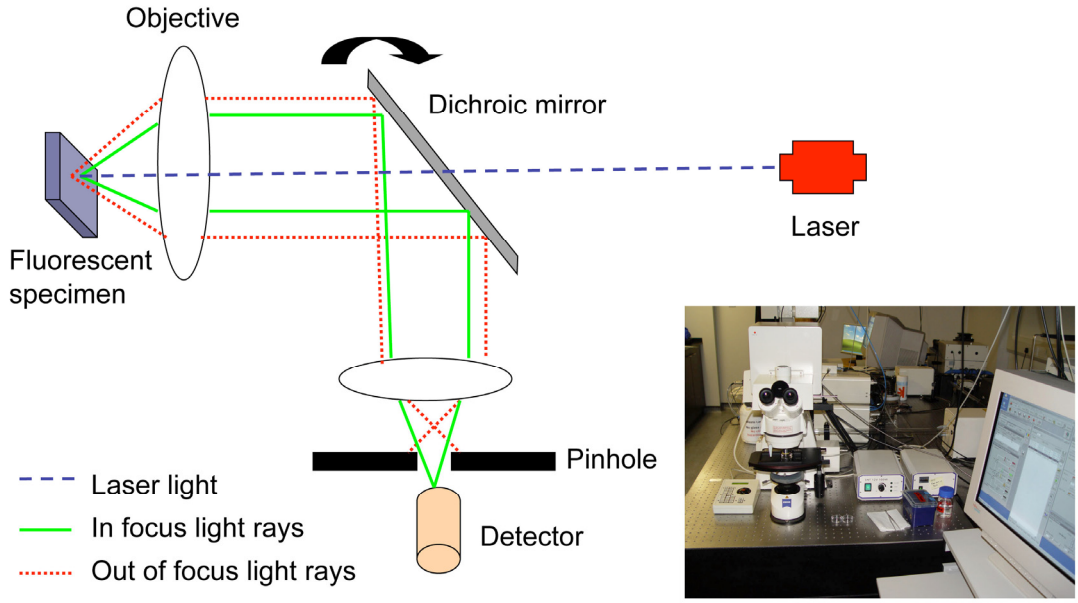


**Figure 1.2: Simplified diagram for the system setup of a confocal microscope illustrating principles of point-by-point illumination and exclusion of out-of-focus light**

Light from the laser reflects off the dichroic mirror and excites one point on the fluorescent labelled specimen. The dye in the specimen is excited by the laser light and fluoresces. The emitted light passes through the dichroic mirror and is focused onto the pinhole and measured by a detector. Out-of- focus light rays are prevented from reaching the detector by the pinhole. The entire specimen is scanned by using a set of rotating mirrors (not shown). Inset shows a photograph of the CLSM set up in the laboratory.

### 1.3.3 CLSM and articular cartilage

The technique of fluorescence-mode CLSM has received considerable attention in cartilage research over the past decade because of its advantages of non-destructive imaging compared with conventional histology (Jones *et al.* 2005). It has been successfully used to characterise healthy and degenerate articular cartilage (Bush & Hall 2005a), study the deformation behaviour and morphology of articular chondrocytes(Guilak, Ratcliffe, & Mow 1995;Poole 1997;Murray *et al.* 2010) and image components of the extracellular matrix such as collagen (Poole, Ayad, & Gilbert 1992). The combination of CLSM and fluorescent probes that label live and dead *in situ* chondrocytes provides a powerful method for investigating articular cartilage subjected to mechanical injury (Jones & Senft 1985;Poole, Brookes, & Clover 2003;Jones *et al.* 2005;Bush *et al.* 2005b;Bush, Wokosin, & Hall 2007). In this work, fluorescence-mode CLSM has been used to define the spatial and temporal patterns of *in situ* chondrocyte death after mechanical injury.

## 1.4 Rationale and aims of the thesis

The rationale for this work is based on developing strategies that decrease the extent of chondrocyte death following mechanical injury to articular cartilage. It is envisaged that the work may provide information of translational relevance to current clinical and experimental approaches to cartilage injury and repair. The objectives of this thesis may be summarised as follows:

1. To develop a reproducible *in vitro* model of mechanical injury to articular cartilage that, in conjunction with fluorescence mode CLSM, allows spatial and temporal quantification of the distribution of *in situ* chondrocyte viability following full thickness mechanical injury to bovine and human articular cartilage.

2. Utilise this model to investigate the effect of different chondroprotective strategies that may decrease the extent of *in situ* chondrocyte death that results from the mechanical injury. Three different putative strategies have been evaluated (a) altering medium osmolarity (b) altering medium calcium concentration and, (c) subchondral bone attachment to articular cartilage. The rationale for choosing these potentially chondroprotective parameters, the applicable hypotheses and a discussion of the pertinent findings are detailed in the relevant chapters.

3. To evaluate whether potentially chondroprotective findings translate to a clinically applicable strategy by serial investigation of clinically relevant parameters in human articular cartilage.

### 1.5 Overview of the chapters

In Chapter 2 - Methods, the first objective of the thesis is addressed with a description of an *in vitro* model of mechanical injury in bovine and human articular cartilage. The technique of CLSM to visualise *in situ* chondrocyte viability after mechanical injury in three- dimensions and the related methods for quantitative analyses are described. All additional materials and methods used to perform experiments are also detailed.

Chapters 3-8 comprise the main results section and encompass all of the objectives of the thesis. In Chapter 3, the effect of varying medium osmolarity on the spatial and temporal distribution of *in situ* chondrocyte death is investigated in mechanically injured bovine articular cartilage. In Chapter 4, the effect of varying medium calcium concentration on the spatial and temporal distribution of *in situ* chondrocyte death is investigated in mechanically injured bovine articular cartilage. In Chapters 5 and 6, key elements of the chondroprotective strategies established in Chapters 3 and 4 are extended to determine whether the findings translate to a clinically applicable strategy with serial investigation in injured bovine (Chapter 5) and human (Chapter 6) articular cartilage.

The experiments detailed in Chapters 7 and 8 evaluate the influence of subchondral bone on *in situ* chondrocyte survival in bovine and human articular cartilage. These experiments were not initially envisaged, but arose from intriguing observations of the lack of secondary cell death in injured cartilage in earlier experiments and are considered to be of relevance to the objectives of this thesis as well as to future research into cartilage injury and repair.

In Chapter 9, the merits and limitations of this work are discussed. In addition, the translational relevance of the findings to experimental and clinical research is detailed. In Chapter 10, preliminary experiments performed to guide future directions of research are outlined.

Finally, the Appendix includes copies of five peer-reviewed, published papers. Chapters 3, 4, 5, 6 and 7 form the basis for each of the five papers. The Appendix also includes a list of published abstracts, presentations, manuscripts in preparation and a copy of the letter of ethical permission for the use of human tissue related to this work.

# CHAPTER 2

# METHODS

## 2.1 BIOCHEMICALS

All biochemicals were obtained from Invitrogen Ltd (Paisley, United Kingdom), with the following exceptions: Formaldehyde solution (10% v/v in normal saline; pH 7.3) was obtained from Fisher Scientific (Leicestershire, United Kingdom). Saline (0.9%, $[Na^+]$ = 154mM, $[Cl^-]$ = 154 mM) was obtained from Baxter Healthcare Ltd (Norfolk, UK). Hartmann's solution ($[Na^+]$ = 131 mM, $[Cl^-]$ = 111mM, $[HCO_3^-]$ = 29 mM, $[K^+]$ = 5 mM, $[Ca^{2+}]$ = 2 mM) was obtained from Fresenius Kabi Ltd (Cheshire, UK).

### 2.1.1 Culture media

The standard culture medium was serum-free Dulbecco's Modified Eagle's Medium (DMEM, 340 mOsm/kg $H_2O$, pH 7.4) buffered with N—hydroxyethylpiperazine-N'-2-ethane sulphonic acid or sodium bicarbonate and containing 4500mg/L glucose, penicillin (50U/ml) and streptomycin (50μg/ml). For experiments performed in Chapter 4, the DMEM was identical but with a slightly lower osmolarity (330 mOsm/kg $H_2O$) as it did not contain any added calcium chloride (Catalogue No. 21068028). Since there were no other calcium containing inorganic salts in this proprietary preparation of DMEM without added calcium chloride, it has been referred to as 'calcium-free (0 mM)' media in subsequent sections of the thesis, although it is accepted that a nominal amount of free calcium is still present (due to a carry over of the water used to formulate the media) and medium calcium concentration for this particular DMEM preparation therefore approaches 0 mM.

Serum-free DMEM was used in all experiments for two primary reasons. Firstly, it was important that the concentration of all ionic and non-ionic constituents of the culture media were known – the media was not supplemented, for instance, with fetal calf serum (FCS), to avoid undefined alterations in the ionic composition of the culture media that may occur with its addition. Secondly, the effects of endogenous bone-mediated factors that may influence chondrocyte survival were important for some of the experiments and could be assessed without the potentially confounding effects of exogenous supplemental nutrient preparations.

### 2.1.2 Measuring medium osmolarity

The osmolarity of all prepared solutions was measured using a freezing point osmometer (Advanced Micro Osmometer, Model 3300, Vitech Scientific Ltd, West Sussex). Three measurements were taken for each prepared solution and the mean osmolarity value rounded to the nearest multiple of five. For example, the osmolarity of the DMEM used for the experiments was measured using three different samples as 342 mOsm, 340 mOsm and 343 mOsm. The mean measured osmolarity of DMEM was therefore 341.7 mOsm and is reported as 340 mOsm. Similarly, the measured osmolarities of the proprietary (control) solutions of 0.9% Saline and Hartmann's were 285 mOsm and 255 mOsm respectively.

### 2.1.3 Measuring medium protein content

The culture medium protein content was measured where relevant to detect changes in the composition of the media which could have potentially influenced chondrocyte survival. It was measured using the Bio-Rad Protein assay (Bio-Rad Laboratories,

Hertfordshire, United Kingdom) with lyophilised bovine serum albumin as the standard. This is a colorimetric assay for measuring total protein concentration (Bradford 1976). It involves the addition of an acidic dye (Coomassie Brilliant Blue G-250) to a protein solution, a colour change of the dye in response to various concentrations of protein as it binds to primarily basic (especially arginine) and aromatic amino acid residues and subsequent measurement of absorbance with a spectrophotometer. Comparison to a standard curve provided a relative measurement of protein concentration.

The lyophilized bovine serum albumin standard was reconstituted by adding 20 mls of de-ionized water and mixing until dissolved. The micro-assay protocol was used for samples as the concentrations of protein were <25 µg/ml. Briefly, five dilutions of the protein standard were prepared representative of the protein solutions to be tested. The standard and sample solutions (800 µl of each) were added to a clean, dry test tube using a pipette. Next, 200 µl of dye reagent concentrate was added to each tube, mixed and the samples incubated at room temperature for 5 minutes. All protein solutions were assayed in duplicate. Absorbance was measured with a spectrophotometer set at 595 nm and the concentration of protein within the solutions determined by comparison with the standard curve. The total protein content in the culture medium was then standardised to wet weight of tissue and expressed as µg protein/mg wet tissue.

### 2.1.4 Varying medium osmolarity

Medium osmolarity was increased by adding measured amounts of sodium chloride (Chapter 3) or sucrose (Chapters 4 and 5) to the solutions. Although the metabolic effects on chondrocytes are similar for both osmolytes (Urban, Hall, & Gehl 1993), sucrose was preferred in later experiments as it minimises intracellular ionic perturbations and is known to maintain an extracellular osmotic pressure gradient without being metabolised by articular chondrocytes (Fell 1969;Fell & Dingle 1969) – the effects of the two osmolytes are considered further in Chapter 5, Section 5.5. Medium osmolarity was decreased by adding measured volumes of distilled water to the solutions. The osmolarity of the solutions was varied between 0 to 600 mOsm.

### 2.1.5 Varying medium calcium concentration

Medium calcium concentration was varied from 0-20 mM by adding calcium chloride (1M stock solution) and the medium osmolarity corrected to 330 mOsm by titrating calculated volumes of a 1M sucrose solution. This was because the osmolarity of the proprietary ‘calcium free’ DMEM was 330 mOsm and the addition of calcium chloride to the DMEM would increase the osmolarity of the prepared solutions. To control for this potentially confounding variable, the osmolarity of all prepared solutions in Chapter 4 was maintained at 330 mOsm. This was achieved by initially decreasing the osmolarity of DMEM from 330 mOsm to 280 mOsm by adding distilled water. The initial dilution of the culture media ensured that the ionic concentration of all solutes was decreased by the same factor prior to preparation of osmotically corrected (sucrose added), calcium-rich media. The proprietary DMEM obtained from the manufacturer (Catalogue No. 21068028) contained sodium

bicarbonate buffer at a concentration of 3700 mg/L. This relatively high concentration of bicarbonate in the culture medium was desirable, due to its key role in the maintenance of normal intracellular pH in chondrocytes (Simpkin *et al.* 2007). However, the presence of bicarbonate in the culture medium does have the potential to reduce ionised calcium concentrations, for example due to the crystallisation of calcium carbonate. Although the actual ionised calcium concentrations in the experimental media was not measured, it is likely that this effect is negligible as bicarbonate at the millimolar concentrations used here, does not significantly alter ionised calcium levels (Zhu *et al.* 2005)**.**

### 2.1.6 Fluorescent probes

A two-fluorophore viability assay was employed to identify live and dead cells:

1. LIVE CELLS: The fluorescent probe, 5- chloromethylfluorescein diacetate (CMFDA), was used to identify live *in situ* chondrocytes. CMFDA passes freely through the cell membrane, but once inside the cell, is cleaved by intracellular esterase to produce a fluorescent and membrane-impermeant product (5-chloromethylfluorescein) which stains the cytoplasm of live cells green (Poole *et al.* 2003). CMFDA was prepared in dimethyl sulfoxide (DMSO) as a 1 mM stock solution.
2. DEAD CELLS: The fluorescent probe, Propidium iodide (PI), was used to identify dead *in situ* chondrocytes. PI is highly fluorescent when bound to nucleic acids, but being a charged molecule, it is only capable of crossing the plasma membrane of dead cells (Jones *et al.* 1985). PI therefore stains the nuclei of dead

cells red. PI was used an aqueous 1mM stock solution obtained from the manufacturer.

## 2.2 BOVINE ARTICULAR CARTILAGE EXPLANTS

Bovine articular cartilage explants were prepared as rectangular osteochondral (articular cartilage with subchondral bone attached) and chondral (articular cartilage with subchondral bone excised) blocks with mechanically injured (scalpel cut) cartilage edges. The technique of preparing these explants is detailed below.

### 2.2.1 Source of bovine tissue

The metacarpophalangeal joints from different, three-year old cows were skinned, rinsed in water and opened under sterile conditions within 12 hours of slaughter. The joint was initially stabilized on a metal clamp, the distal limb reflected to expose the articular surface and the synovial fluid allowed to drain from the joint (**Figure 2.1**). Particular care was taken throughout the exposure to avoid mechanical damage to the articular surface during dissection.

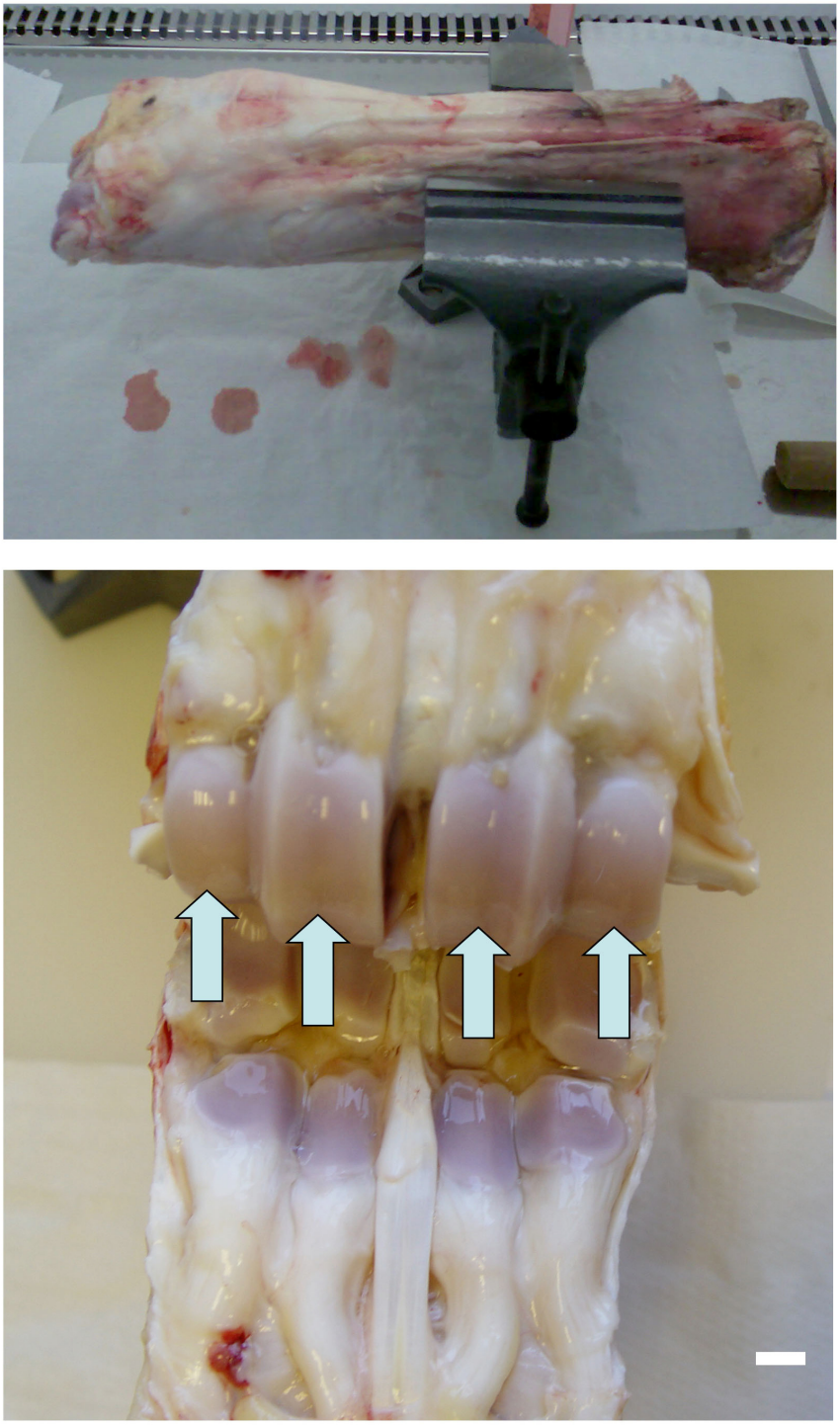

**Figure 2.1: Exposing the bovine metacarpophalangeal joint**

The joint has been stabilised on a clamp (top figure). It is then opened by reflecting the distal limb to expose the proximal articular surface (block arrows, bottom figure) used for cartilage harvest (white bar = 1 cm).

### 2.2.2 Initial removal of osteochondral 'strips'

Three different instruments were initially tested for harvesting osteochondral strips from the flat articular surface intervening the condylar ridges of each bovine metacarpophalangeal joint: (1) No. 24 Scalpel (2) Hand-held saw blade (Addis, Sheffield, UK) (3) Chisel and 225g hammer. The scalpel blade produced small osteochondral 'flakes' with little attached subchondral bone. The saw cut through subchondral bone adequately but produced metal debris during cutting. The chisel and 225g toffee hammer allowed consistent harvest of osteochondral strips measuring approximately 1 cm x 2 cm with 2-3mm of attached subchondral bone without producing metal debris and were therefore the preferred instruments for osteochondral strip harvest (**Figure 2.2 A-B**). The osteochondral strips were immediately placed in DMEM, maintained in an incubator at 37°C (5% $CO_2$, pH 7.4) and used within 30 minutes except for the experiments performed in Chapter 4, where the osteochondral strips were pre-incubated in calcium-free (0 mM, controls) and calcium-rich (2-20 mM) DMEM for 12 hours at 37°C (5% $CO_2$, pH 7.4), to allow tissue equilibration with the culture media before experimental mechanical injury.

### 2.2.3 Mechanically injured osteochondral explants

Osteochondral explants (rectangular blocks) with mechanically injured (scalpel cut) cartilage edges were obtained from osteochondral strips using the following standardized technique. First, any irregular osteochondral edges around the periphery of each strip were cut with a No. 24 scalpel to produce a 'trimmed' osteochondral strip with four straight edges. At this stage, if exposure to different media (medium osmolarities ranging between 0-600 mOsm or calcium concentrations ranging

between 0-20 mM) was relevant for the experiment, osteochondral strips were exposed to the various media for 90-120 seconds prior to the mechanical injury to allow *in situ* chondrocytes to experience and respond to the altered external environment (Bush & Hall 2001b). Next, the articular cartilage on each trimmed osteochondral strip was injured through its full thickness with a fresh No. 24 scalpel (**Figure 2.2 C-D**). No rotational or sliding movement was made during scalpel injury with the cartilage 'cut' in push-through mode, and the direction of the cutting force applied solely perpendicular to the blade edge. The wound was deepened through subchondral bone by gentle tapping of the scalpel blade with a 225g toffee hammer. The first and the last explants were discarded as the outer edges of these explants were used to handle the osteochondral strip and could have been subjected to an additional mechanical force other than that associated with the scalpel injury. Hence, two or three osteochondral explants with mechanically injured (scalpel cut) cartilage edges were harvested as rectangular blocks from each strip (**Figure 2.2 E**). As the long cut edges of each explant were of interest for the experiment, a strict 'no-touch' technique was employed with explants handled only from the short edges. Articular cartilage is an anisotropic tissue (Jeffery *et al.* 1991). Therefore, for standardisation each scalpel wound was made in the same direction (perpendicular to the long axis of the trimmed osteochondral strip). A fresh No.24 scalpel was used for every scalpel wound and discarded after single use because of concerns regarding the retention of blade sharpness (Huntley *et al.* 2005b). Due to the adverse effects of drying on chondrocytes (Mitchell & Shepard 1989), osteochondral strips and explants were kept wet at all times with their respective incubating media.

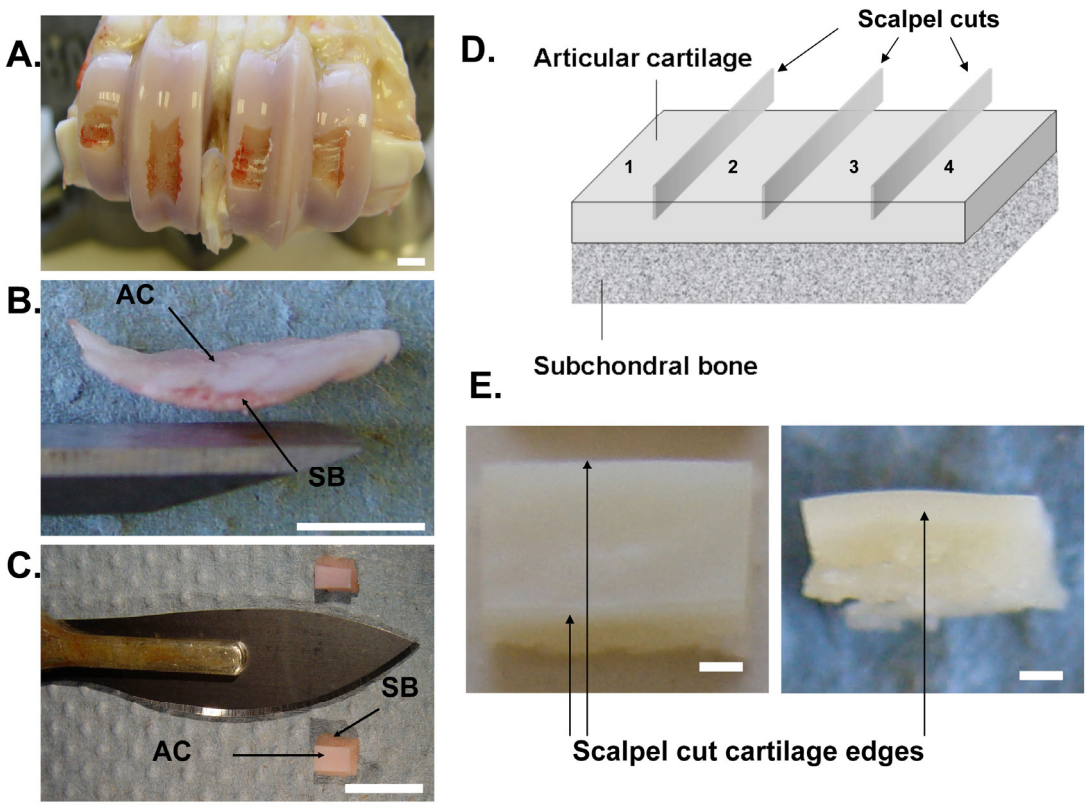


**Figure 2.2: Bovine osteochondral explants with mechanically injured (scalpel cut) cartilage edges**

**A.** Osteochondral strips harvested from the flat articular surface between the condylar ridges of a bovine metacarpophalangeal joint (white bar = 1 cm). **B.** Lateral view of an osteochondral strip harvested using the chisel showing articular cartilage (AC) and subchondral bone (SB, white bar = 1 cm). **C.** Two scalpel injured osteochondral explants harvested as rectangular blocks from an osteochondral strip (white bar = 1cm). **D.** Diagram of scalpel injury – a trimmed osteochondral strip (with irregular edges cut to produce four straight edges) was injured with a fresh No. 24 blade perpendicular to its long axis to produce osteochondral explants (2 and 3) as rectangular blocks. The first (1) and last (4) explants were discarded (see section 2.2.3 for details). **E.** Magnified images of a bovine osteochondral explant (rectangular block) with scalpel cut cartilage edges viewed top-down (left image) and side-on (right image). The long cartilage edges were of interest and subsequently imaged using CLSM. The explant was handled only from the short edges (white bar = 1 mm).

### 2.2.4 Mechanically injured chondral explants

Chondral explants (articular cartilage with subchondral bone excised) were prepared for experiments investigating the role of subchondral bone within a co-culture model (Chapter 7). These chondral explants were obtained by carefully placing a rectangular osteochondral explant on its short edge and dividing the explant at the bone-cartilage junction with a No. 11 scalpel. Using forceps, care was taken to hold only the osseous component of the osteochondral explant during excision of cartilage from bone. With this technique, the chondral explant was also harvested as a rectangular block with mechanically injured (scalpel cut) long cartilage edges. The chondral explant was handled only from its short edges using a strict 'no touch' technique that avoided any additional mechanical insult to the long cartilage edges of interest for the experiments. The excised block of subchondral bone from the explant was utilised for subsequent co-culture if required. Hence, osteochondral and chondral explants from the same bovine metacarpophalangeal joint were placed into three distinct groups: (1) subchondral bone excised from articular cartilage (Group A), (2) subchondral bone left attached to articular cartilage (Group B), (3) subchondral bone excised from, but co-cultured with articular cartilage (Group C, **Figure 2.3**). The scalpel cuts used to produce the rectangular blocks of explants were made using the same protocol so that a uniform region of mechanical injury was created through the full thickness of cartilage.

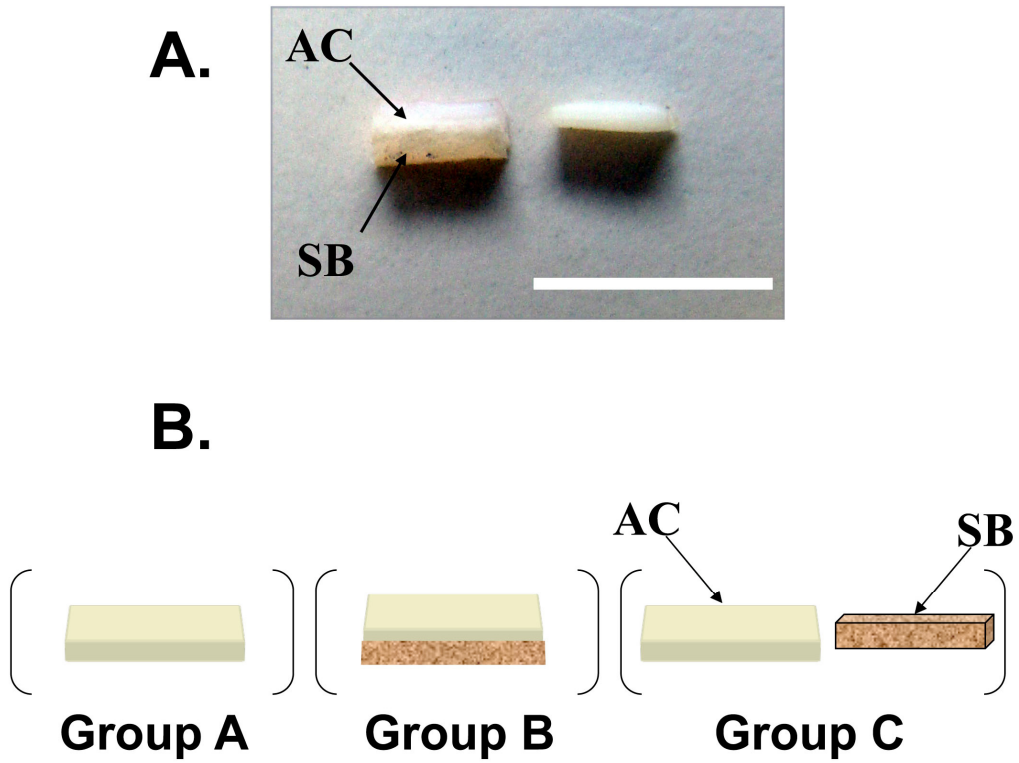


**Figure 2.3: Bovine chondral and osteochondral explants for co-culture**

**A.** Rectangular blocks of osteochondral (cartilage with attached subchondral bone) and chondral (cartilage with subchondral bone excised) explants.

**B.** Diagram of the three experimental groups with either subchondral bone excised from articular cartilage (Group A), subchondral bone left attached to articular cartilage (Group B) or subchondral bone excised from, but co-cultured with articular cartilage (Group C, AC=Articular cartilage, SB=Subchondral bone, white bar = 1 cm).

## 2.3 HUMAN ARTICULAR CARTILAGE EXPLANTS

Human articular cartilage explants were also prepared as rectangular blocks with mechanically injured (scalpel cut) cartilage edges. The technique for preparing these explants was similar to that described for bovine tissue and is outlined below.

### 2.3.1 Source of human tissue

At total knee replacement, the distal femoral and proximal tibial articular surfaces of the human knee joint are normally resected as separate, thick osteochondral cuts. Ethical approval for the use of such human osteochondral tissue discarded at total knee replacement was obtained from the Local Research Ethics Committee (please see Appendix III for letter of ethical approval). In addition, informed patient consent for the use of the material was obtained from 48 patients undergoing total knee replacement for osteoarthritis. All osteochondral tissue obtained from the resected femoral and tibial condyles of the human knee joint was immediately placed in serum-free DMEM and utilised within six hours following resection from the native joint. A protocol of utilising the tissue for experiments within six hours was strictly followed in order to avoid loss in cartilage cellularity that may occur during longer storage in culture medium (Williams *et al.* 2003;Pennock *et al.* 2006a).

### 2.3.2 Initial removal of osteochondral 'strips'

Of the 48 patients from whom osteochondral tissue was obtained at total knee replacement for osteoarthritis, regions of articular cartilage that appeared non-degenerate following an initial macroscopic visual assessment were identified on the femoral or tibial condyle of 14 patients (**Figure 2.4A,** see section 2.5.3 for details

regarding microscopic verification of non-degenerate cartilage). Osteochondral strips measuring approximately 1 cm x 2 cm were cut from these regions with a No.24 scalpel (**Figure 2.4B**).

### 2.3.3 Mechanically injured osteochondral explants

Osteochondral strips from the human knee joint were approximately 5 mm thicker than similar tissue obtained from the bovine metacarpophalangeal joint. However, the cartilage and underlying subchondral bone could be cut easily using a No.24 scalpel blade. Osteochondral explants (rectangular blocks of articular cartilage with subchondral bone attached) with mechanically injured (scalpel cut) cartilage edges were obtained from osteochondral strips using the standardised technique described earlier for bovine tissue (**Figure 2.4C**). If exposure to different media was relevant for the experiment, osteochondral strips were exposed to these solutions for five minutes prior to the scalpel injury to allow *in situ* chondrocytes to experience and respond to the altered external environment (Bush *et al.* 2005a).

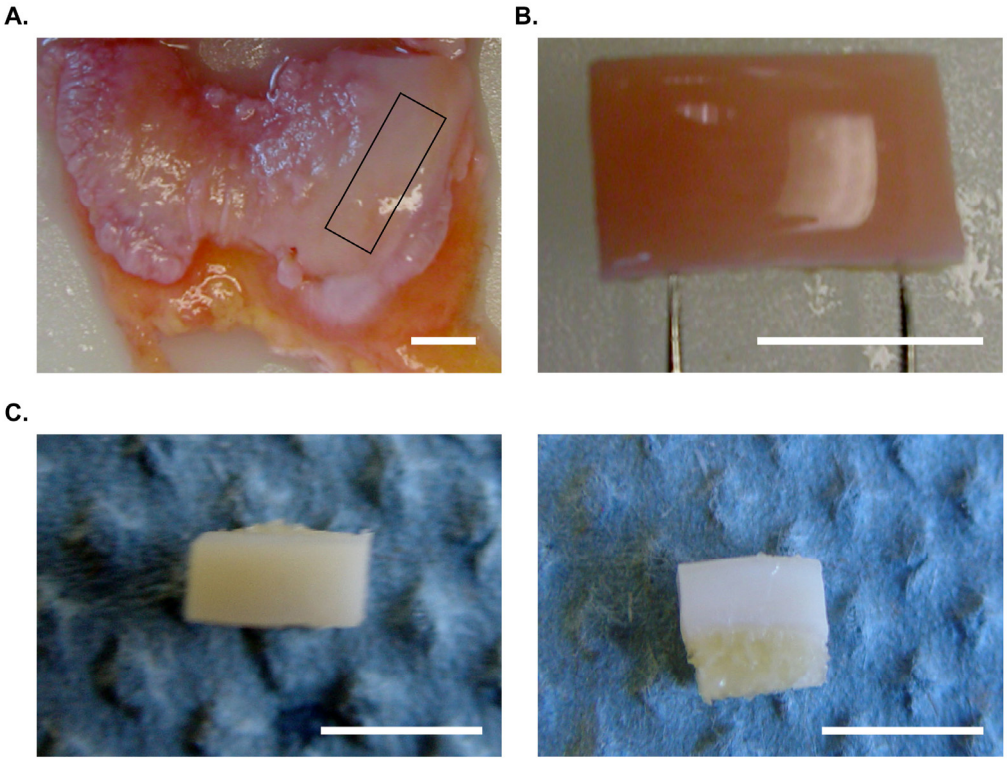


**Figure 2.4: Human osteochondral explants with mechanically injured (scalpel cut) cartilage edges**

**A.** Non-degenerate articular cartilage (boxed) present on a resected femoral condyle discarded during total knee replacement for osteoarthritis.

**B.** Osteochondral strip cut out from the non-degenerate region of the femoral condyle (two from each femoral condyle)**.**

**C.** Magnified images of a human osteochondral explant (rectangular block) with scalpel cut cartilage edges viewed top-down (left image) and side-on (right image). The long cartilage edges were of interest and subsequently imaged using CLSM. Note the human articular cartilage is thicker on the explants compared with bovine explants shown in **Figure 2.2E** (white bar = 1 cm).

### 2.3.4 Mechanically injured chondral explants

Human chondral explants (rectangular blocks with subchondral bone excised from articular cartilage) with mechanically injured (scalpel cut) edges were obtained from osteochondral strips using the standardised technique described earlier for bovine tissue (see section 2.2.4). The chondral explants were specifically prepared for experiments investigating the role of subchondral bone within a co-culture model (Chapter 8). In these explants, the excised rectangular bone blocks represented healthy subchondral bone and were used for co-culture with the respective chondral explants from the same knee. To identify sclerotic subchondral bone, pre-operative radiographs were studied to identify the diseased compartment of the knee (lateral or medial). With further macroscopic evaluation of the tissue, it was relatively easy to identify regions of sclerotic subchondral bone within the resected specimens as cartilage loss occurs in the same subregion as subchondral bone sclerosis (Neogi *et al.* 2009a;Neogi *et al.* 2009b). Rectangular blocks of sclerotic subchondral bone from the diseased compartment of the same knee were then cut from these regions using a hand held saw.

## 2.4 INCUBATION, CELL VIABILITY STAINING AND FIXATION

Explants were either incubated for 2.5 hours or cultured over 7 days. Explants were incubated for 2.5 hours in 1 ml media (DMEM or prepared solution) at 37°C (5% $CO_2$, pH 7.4). Explants were cultured over 7 days in 8 mls of DMEM at 37°C (5% $CO_2$, pH 7.4) with alternate daily media changes. During explant co-culture (Chapters 7 and 8), rectangular blocks of bone and cartilage were placed in the same well with further incubation over 7 days at 37°C (5% $CO_2$, pH 7.4). For these

experiments, explants were cultured in 1 ml of DMEM over 7 days without media changes - the lower volume of DMEM was used to optimise potential biochemical interactions between the tissues within the co-culture system.

To determine cell viability, explants were exposed to CMFDA (5 or 10μM) and PI (5 or 10μM) during the final 30-45 minutes of incubation or culture to label live and dead cells, respectively. Following labelling, explants were washed in DMEM to remove excess dye, transferred to 10% formalin (v/v in saline) for fixation and stored at 4°C in phosphate buffered saline in preparation for microscopy. The products of staining with CMFDA and PI are covalently linked to biomolecules after formaldehyde fixation, permitting storage of labelled explants before microscopy (Poole *et al.* 2003). Containers were wrapped in tinfoil during storage to prevent dye loss from exposure to ultraviolet light.

## 2.5 CONFOCAL LASER SCANNING MICROSCOPY (CLSM)

Explants were anchored to the base of a plastic dish with Blu-Tack (Bostik Ltd, Leicester, UK) with the articular surface uppermost and analysed in phosphate buffered saline. An upright Zeiss Axioskop LSM 510 confocal laser scanning microscope fitted with a low-power (x10 dry) objective was used to acquire optical sections (512 x 512 pixels) of fluorescently labelled *in situ* articular chondrocytes. A multi-track protocol involving Argon and Helium-Neon laser excitation, band-pass filters (500-550 nm) and long-pass filters (>560 nm) allowed separation and measurement of the fluorescence emitted from CMFDA (λexcitation (ex)=488 nm, λemission (em)=517 nm) and PI (λex=543 nm, λem=650 nm) respectively.

### 2.5.1 CLSM – bovine articular cartilage

By moving the focal plane sequentially through the depth of bovine articular cartilage, a consecutive series of 921 x 921 $\mu m^2$ optical sections were acquired at 10 μm intervals near the injured edge of the rectangular cartilage explants in two orthogonal planes

1. Coronal plane (x-z axes): these optical sections imaged the full thickness of cartilage and were acquired at the scalpel cut surface to a depth of ~ 80 μm into the tissue (y-axis). The heterogeneous spatial distribution of *in situ* chondrocytes was clearly visible in this plane - parallel to the articular surface in the superficial zone and more vertically in columns in the middle and deep zones (**Figure 2.5**).
2. Axial plane (x-y axes): these optical sections imaged the superficial zone 'top-down' from the articular surface and were acquired near the cut edge of the articular surface to a depth of ~ 60 μm into the tissue (z-axis). *In situ* chondrocytes at the articular surface were clearly visible in this plane. The injured (scalpel cut) cartilage was seen as a band of red-stained nuclei at its cut edge (**Figure 2.5**).

By imaging the scalpel cut edges of articular cartilage in coronal and axial planes, *in situ* chondrocyte death was spatially defined within cartilage from 'full thickness' and 'articular surface' perspectives. Three-dimensional coronal and axial CLSM reconstructions of the imaged volume of articular cartilage were created from the consecutive series of optical sections using imaging software (Volocity 4.0, Improvision, Coventry, UK). These CLSM reconstructions provided a composite view of live and dead *in situ* articular chondrocytes within the entire injured zone of cartilage (**Figure 2.5**).

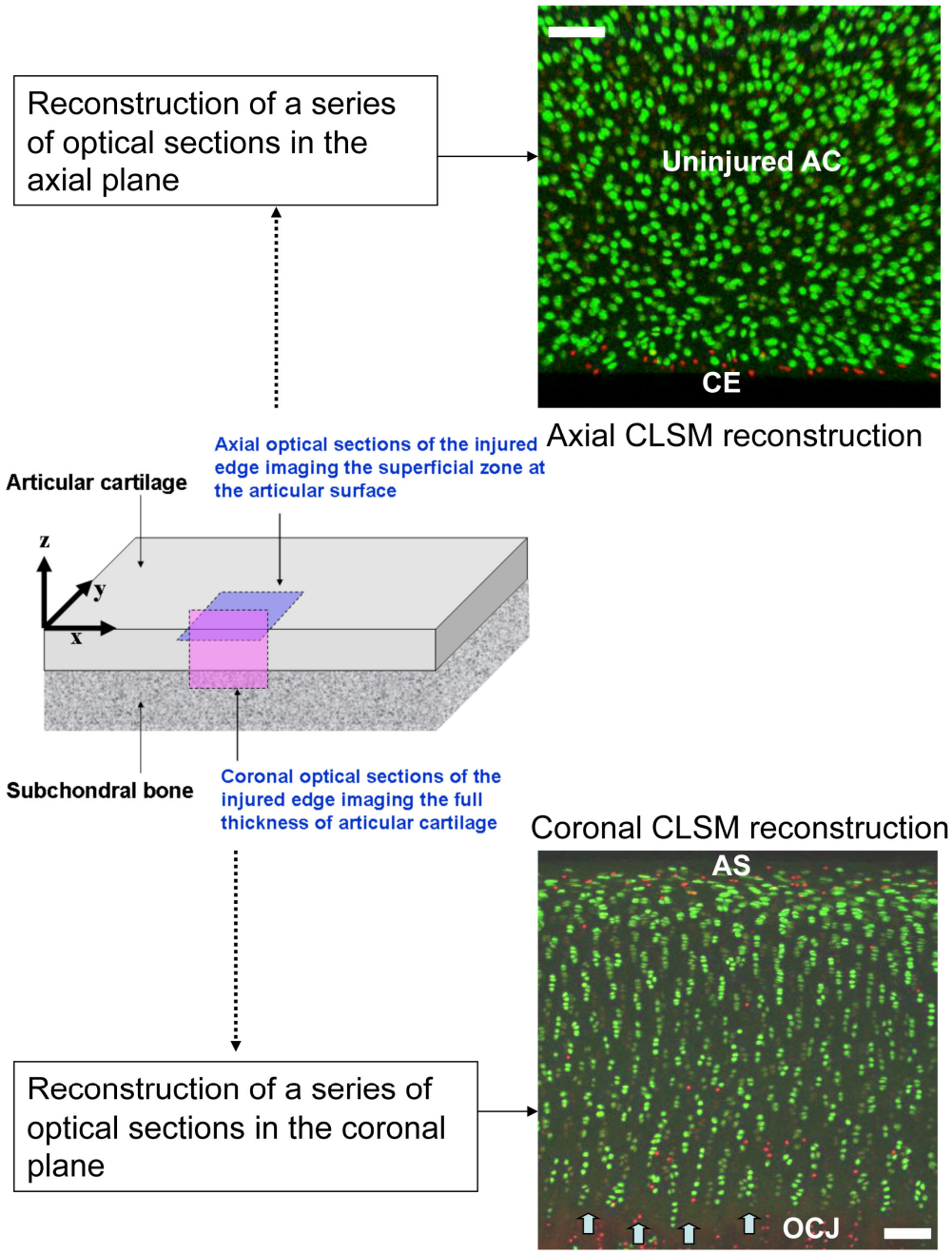


**Figure 2.5 Coronal and axial CLSM for injured bovine articular cartilage explants**

The x, y, and z axes are labelled on a diagram of an explant. Coronal optical sections (pink-shaded area) imaged the full thickness of cartilage. In the coronal CLSM reconstruction, note the articular surface (AS), the osteochondral junction (OCJ) and the tidemark (block arrows). Axial optical sections (blue-shaded area) imaged the AS top-down at the cut edge (CE) of cartilage. In the axial CLSM reconstruction, note the band of cell death at the CE and viable cells in the adjacent uninjured articular cartilage (AC) (PI stains dead cells red, CMFDA stains live cells green, white bar=100 μm).

Fluorescence was poor at depths greater than 60 μm in the axial plane and depths greater than 80 μm in the coronal plane. These depths into cartilage were therefore taken as the limits for image acquisition. The optical sections were acquired only from the central region of the long edges of each explant as chondrocyte death at the short edges overestimated total cell death at the corners of the explants. Laser power, detector gain, and sensitivity were adjusted to obtain optimal image quality without excessive dye bleaching or saturation. In summary, the important features in the CLSM reconstructions of mechanically injured bovine articular cartilage were as follows:

Coronal CLSM reconstructions

1. The full thickness of the articular cartilage (from the articular surface to the tidemark) visible within a single coronal CLSM reconstruction.
2. Live/dead responses of *in situ* articular chondrocytes within different zones (superficial, middle and deep) visualised at the scalpel cut surface.

Axial CLSM reconstructions

1. The cut edge of cartilage visualised top-down from the articular surface showing the band of *in situ* chondrocyte death (in the superficial zone) from mechanical (scalpel) injury at the cartilage edge.
2. The adjacent uninjured region of articular cartilage visualised in the same image allowing assessment of any (a) progression of cell death into the unaffected cartilage, (b) potential deleterious effects of different media used during the experiments within normal cartilage and (c) additional cell death in normal cartilage that may be attributed to explant preparation and handling.

### 2.5.2 CLSM – human articular cartilage

By moving the focal plane sequentially through the depth of human articular cartilage, a consecutive series of 921 x 921 $\mu m^2$ optical sections were acquired at 10 μm intervals near the injured edge of the rectangular cartilage explants in two orthogonal planes:

1. Coronal plane (x-z axes): these optical sections were acquired at the scalpel 'cut surface' to a depth of ~ 100 μm into the tissue (y-axis). Since human articular cartilage from the knee joint is 2-4 mm thick, the full thickness of the cut surface of the cartilage could not be imaged (at x10 magnification) within a single coronal optical section measuring 921 x 921 $\mu m^2$. Hence, the coronal optical sections were acquired at three different intervals from the articular surface and visualised *in situ* chondrocytes separately within the superficial, middle and deep zones (**Figure 2.6**). Further, the coronal optical sections were acquired at defined distances from the articular surface to control for the variation in cartilage thickness between explants: superficial zone optical sections were acquired downwards from the articular surface, middle zone optical sections were acquired following an interval of 200 μm and deep zone optical sections were acquired at a distance of 100 μm from the bone-calcified cartilage junction.
2. Axial plane (x-y axes): these optical sections imaged the superficial zone 'top-down' from the articular surface and were acquired near the cut edge of the articular surface to a depth of ~ 100 μm into the tissue (z-axis, **Figure 2.6**).

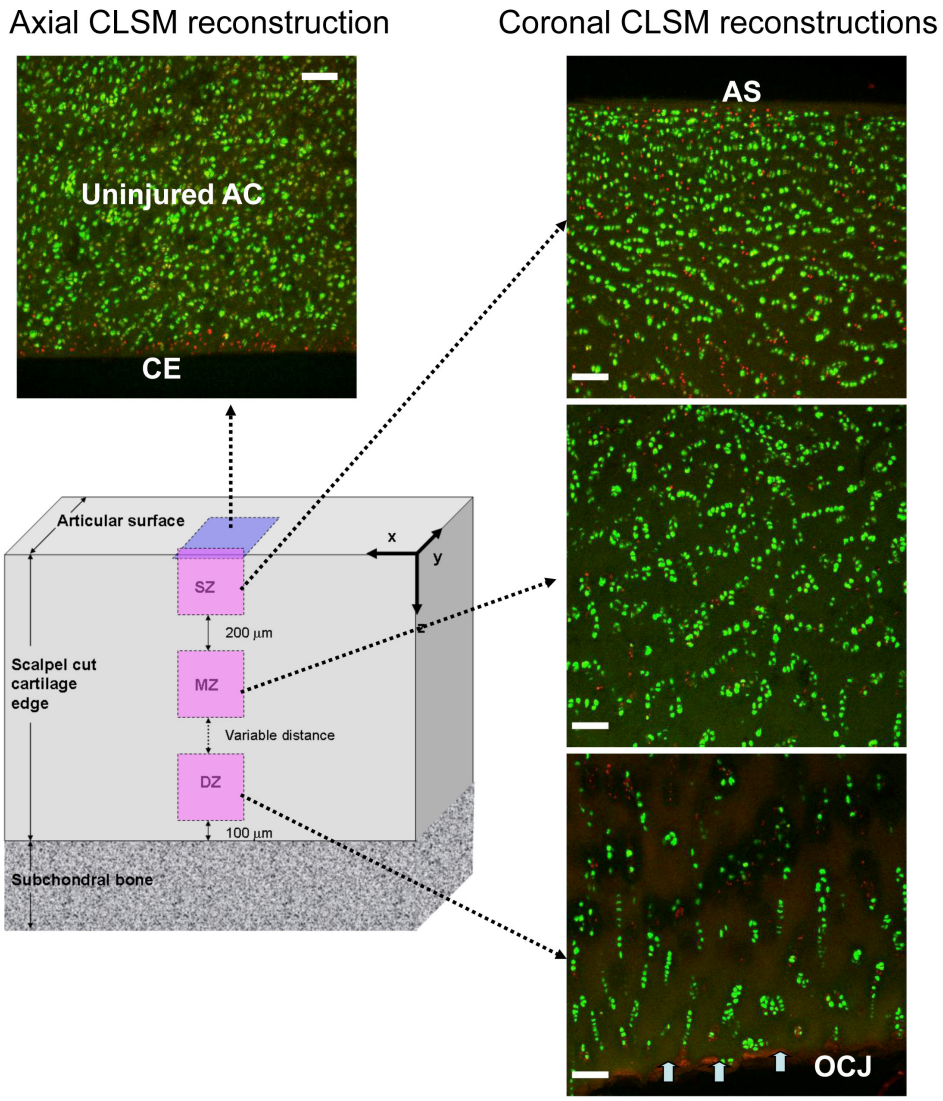


**Figure 2.6: Coronal and axial CLSM for injured human articular cartilage explants**

The x, y, and z axes are labelled on a diagram of an explant. Coronal optical sections (pink-shaded area) imaged the superficial zone (SZ), middle zone (MZ) and deep zone (DZ) of injured cartilage at defined distances for standardisation. In the coronal CLSM reconstructions, note the heterogeneous spatial distribution of *in situ* chondrocytes - cells in the SZ are orientated parallel to the articular surface (AS), whereas cells in the MZ and DZ are orientated more vertically in columns nearing the osteochondral junction (OCJ) and tidemark (block arrows). Axial optical sections (blue-shaded area) imaged the AS top-down at the cut edge of cartilage. In the axial CLSM reconstruction, note the band of cell death at the cut edge (CE) and viable cells within adjacent uninjured articular cartilage (AC) (PI stains dead cells red, CMFDA stains live cells green, white bar = 100 µm).

Three-dimensional coronal and axial CLSM reconstructions of the imaged volume of articular cartilage were created from the consecutive series of optical sections using imaging software (Volocity 4.0, Improvision, Coventry, UK). Fluorescence was poor in the coronal and axial planes at depths greater than 100 μm into cartilage and this was therefore taken as the limit for image acquisition. The optical sections were acquired only from the central region of the long edges of each explant as chondrocyte death at the short edges overestimated total cell death at the corners of the injured explants. Laser power, detector gain, and sensitivity were adjusted to obtain optimal image quality without excessive dye bleaching or saturation. In summary, the important features in CLSM reconstructions of mechanically injured human articular cartilage were as follows:

Coronal CLSM reconstructions

1. Coronal reconstructions of the superficial zone evaluating the integrity of the articular surface and superficial zone cellularity (indicative of whether cartilage was degenerate or non-degenerate, see the section 2.5.3 for further details).
2. Coronal reconstructions of the middle and deep zones showing the zonal heterogeneity within cartilage with a more vertical arrangement of cells compared to the superficial zone.
3. Live/dead responses of *in situ* articular chondrocytes within different zones (superficial, middle and deep) visualised at the scalpel cut surface.

Axial CLSM reconstructions

1. The cut edge of cartilage visualised top down from the articular surface showing the band of *in situ* chondrocyte death (in the superficial zone) from mechanical (scalpel) injury at the cartilage edge.

2. The adjacent uninjured region of articular cartilage visualised in the same image allowing assessment of any (a) progression of cell death into the unaffected cartilage, (b) potential deleterious effects of different media used during the experiments within normal cartilage and (c) additional cell death in normal cartilage that may be attributed to explant preparation and handling.

### 2.5.3 Microscopic grading of human articular cartilage

Initially, non-degenerate articular cartilage was identified by macroscopic visual assessment. Subsequently, the articular cartilage on explants was also evaluated microscopically (using low power (x10) light and confocal microscopy) to confirm it appeared non-degenerate and ensure consistency in the quality of explants used for the experiments. The International Cartilage Repair Society (ICRS) (Mainil-Varlet *et al.* 2003;Huntley, Simpson, & Hall 2005c) classification system allows grading of the severity of degenerative changes in articular cartilage. The system is based on microscopic assessment of articular surface integrity, superficial zone integrity, cell distribution and the extent of articular surface defects (Mainil-Varlet *et al.* 2003;Huntley *et al.* 2005c) and grades articular cartilage from 0-4 as follows:

Grade 0 = Normal

Grade 1 = Nearly normal - superficial lesions only

Grade 2 = Abnormal - lesions extending down to <50% cartilage depth

Grade 3 = Severely abnormal - lesions extending down to >50% cartilage depth

Grade 4 = Severely abnormal, abrading bone

The coronal CLSM reconstructions allowed grading of the articular cartilage according to the ICRS system (Mainil-Varlet *et al.* 2003;Huntley *et al.* 2005c) before quantitative analyses. All articular cartilage explants assigned an ICRS grade 0 (normal) were included in the experiments. ICRS grade 1 (near normal) degenerate cartilage exhibits a wide variation in microscopic appearance - from minimal disruption of the articular surface with normal superficial zone cellularity to complete loss of superficial zone chondrocytes (Huntley *et al.* 2005c) (**Figure 2.7**). Only those explants which represented 'early' grade 1 changes i.e. minimal fibrillation of the articular surface with preservation of superficial zone cellularity were included for the experiments in this work as such tissue has biophysical parameters consistent with normal cartilage (Huntley *et al.* 2005c). Patients in whom the articular cartilage explants showed more significant degenerative changes microscopically, were excluded from the experiments.

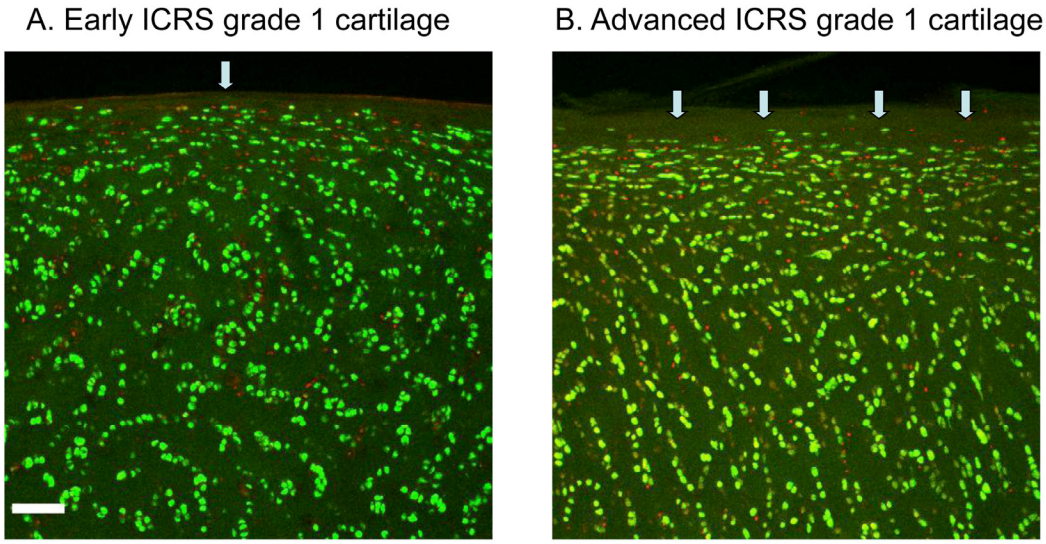


**Figure 2.7: Coronal CLSM reconstructions of the superficial zone of human articular cartilage showing the variability in ICRS grade 1 articular cartilage**

**A.** Early ICRS grade 1 articular cartilage with minor disruption of the articular surface (arrow) but preservation of superficial zone cellularity near the articular surface. Such explants were included in subsequent quantitative analyses.

**B.** More advanced ICRS grade 1 articular cartilage with minor disruption of the articular surface associated with significant loss in superficial zone cellularity (arrows) near the articular surface. Such explants were excluded from subsequent quantitative analyses.

### 2.5.4 Study sample for experiments in human cartilage

Of the 14 patients in whom articular cartilage was judged to be macroscopically normal after visual assessment, significant ICRS Grade 1 or greater changes were present in explants from four patients after microscopic evaluation. These four patients were excluded from further analyses leaving 10 patients from whom tissue was utilised for subsequent quantitative analyses.

For experiments detailed in Chapter 6, ICRS grade 0 articular cartilage was present on explants obtained from four different patients. These four patients forming the study sample for the experiments had a median age of 70 years (range 68–73, two males). For experiments detailed in Chapter 8, ICRS grade 0 articular cartilage was present on explants obtained from four different patients. Additionally in two patients, the articular cartilage represented early ICRS grade 1 degenerative changes (representing minimal fibrillation of the articular surface with preservation of superficial zone cellularity). These two patients were also included in the experiments. The study sample therefore comprised six different patients with a median age of 70 years (range 64-73 years, three males).

## 2.6 QUANTITATIVE ANAYSES

### 2.6.1 Defining the spatial pattern of *in situ* chondrocyte death

The coronal and axial CLSM reconstructions were initially visualised using imaging software (Volocity 4.0, Improvision, Coventry, UK) to establish the spatial pattern of *in situ* chondrocyte death within explants. This allowed appropriate positioning of regions of interest (ROI) on each image for subsequent quantitative analyses. The three-dimensional rendering facility of the imaging software allowed accurate overlay of a ROI of known dimensions in the x, y and z planes of the CLSM reconstructions. This minimised errors that may have otherwise occurred if optical sections were not obtained perfectly parallel to the cartilage surface (due to tilting of the explants) during CLSM (**Figure 2.8**).

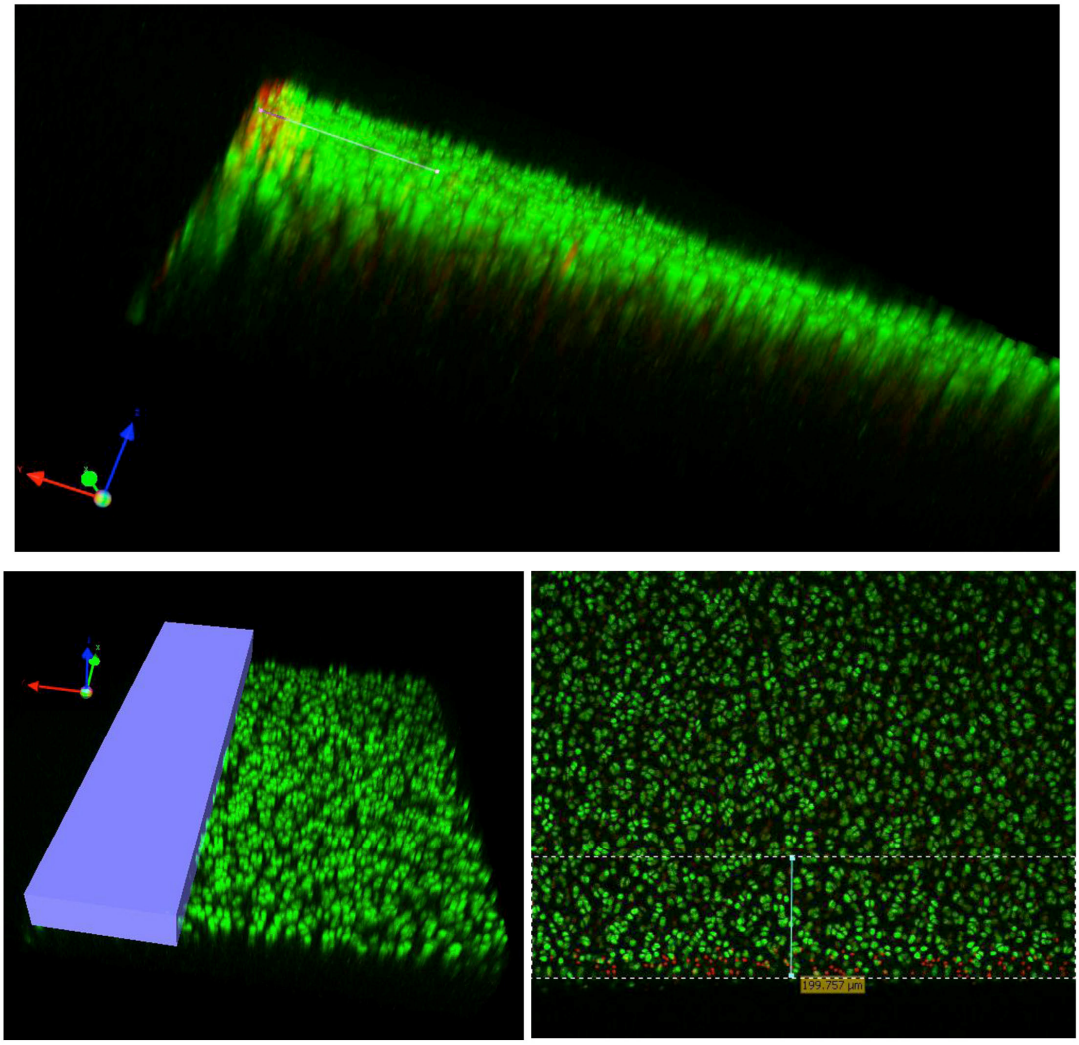


**Figure 2.8: Positioning a ROI in the x, y and z planes of a CLSM reconstruction**

In this example of an axial CLSM reconstruction of bovine articular cartilage, the composite image is 'tilted' in the x-y-z planes (top image, axes labels: x-green, y-red, z-blue). Using imaging software, a line measuring ~200 µm has been placed from the cut edge of the cartilage and extends proximally parallel to the imaged plane of the CLSM reconstruction. This allowed subsequent accurate overlay of the ROI in the x-y-z plane of the image (bottom images, blue box represents ROI, y-axis=200 µm). Note that two dimensional positioning of a ROI on this tilted CLSM reconstruction would be inaccurate in the y-axis, as it would not account for the inclination of the image.

### 2.6.2 Percentage chondrocyte death

Percentage cell death (100 x number of dead cells/ number of dead and live cells) was calculated by counting live (green) and dead (red) *in situ* chondrocytes within ROIs positioned over CLSM reconstructions. Imaging software (Volocity 4.0, Improvision, Coventry, UK) was used for performing automated live/dead cell counts for each CLSM reconstruction. The technique for automated cell counting within each ROI was similar in bovine and human articular cartilage and was based on a validated, reproducible protocol (Jomha *et al.* 2003;Lin *et al.* 2005;Jadin *et al.* 2005) using voxel intensity thresholding as follows: 'Objects' (individual cells) in the green (live) and red (dead) channels within each ROI were identified by thresholding voxel (volumetric pixel) intensity (Lin *et al.* 2005). Percentage thresholds of voxel intensity were set using a histogram of measured values for all objects identified in each channel. The upper limit was 100% with minor adjustments of the lower limit (minimum 5%) to account for variations in cell dye loading, detector sensitivity and noise between images. All cells touching the ROI were included in the counts and combined objects (neighbouring cells in close proximity incorrectly identified as single objects) within the ROI were separated. This protocol returned a list of measured objects in the green and red channels. Using a histogram of measured volumes for all objects identified within each channel, thresholds were set to exclude background noise. In bovine cartilage, when ordered by volume, objects in the green channel $<500\mu m^3$ and in the red channel $<200\mu m^3$, were attributed to background noise and excluded from the cell counts. In human cartilage, when ordered by volume, objects in the green channel $<1000\mu m^3$ and in the red channel $<200\mu m^3$ were attributed to background noise and excluded from the cell

counts. Finally, the entire ROI was visualized in three-dimensions to adjust for any remaining background noise before the software program generated automated live and dead cell counts. Percentage cell death was then quantified for each ROI. Percentage cell death calculations were based on a large number of cells (ranging between 800-2000 individual cells) counted in the axial and coronal plane of each explant. The positioning and dimension of each ROI on a CLSM reconstruction was standardised as follows:

1. Axial CLSM reconstructions

In bovine articular cartilage, percentage cell death was quantified within a ROI measuring 921 x 200 x 60 $\mu m^3$ (x-y-z axes, respectively) or 921 x 500 x 60 $\mu m^3$ (x-y-z axes, respectively) positioned at the injured cartilage edge (**Figure 2.9, Panel A**). The band of cell death at the scalpel cut edge of explants was included in all measurements to evaluate any potential progression of cell death at the wound edge. The smaller ROI with y-axis dimensions of 200 μm was used for experiments in which the extent of cell death at the injured cartilage edge was most relevant (Chapters 3, 4 and 5) from the articular surface perspective. The larger ROI with y-axis dimensions of 500 μm was used for experiments in which *in situ* chondrocyte death occurred in the adjacent uninjured region of the articular surface in order to quantify the increase in cell death within this region of the articular surface (Chapter 7). Similarly in human articular cartilage, percentage cell death was quantified within a ROI measuring 921 x 500 x 100 $\mu m^3$ (x-y-z axes, respectively) positioned at the injured cartilage edge.

2. Coronal CLSM reconstructions

Due to the zonal heterogeneity present in articular cartilage, the viability of *in situ* chondrocytes was also quantified as a function of increasing depth from the articular surface. In bovine articular cartilage, percentage cell death was quantified at 100 μm intervals from the articular surface downwards within a ROI measuring 921 x 80 x 100 $\mu m^3$ (x-y-z axes, respectively, **Figure 2.9 Panel B**). The limit for measurements in this plane was taken as 400μm from the articular surface as the ROI overlapped with the tidemark at greater depths from the articular surface. Similarly in human articular cartilage, percentage cell death was quantified within a ROI measuring 921 x 100 x 500 $\mu m^3$ (x-y-z-axes, respectively) positioned on coronal CLSM reconstructions of the superficial, middle and deep zones.

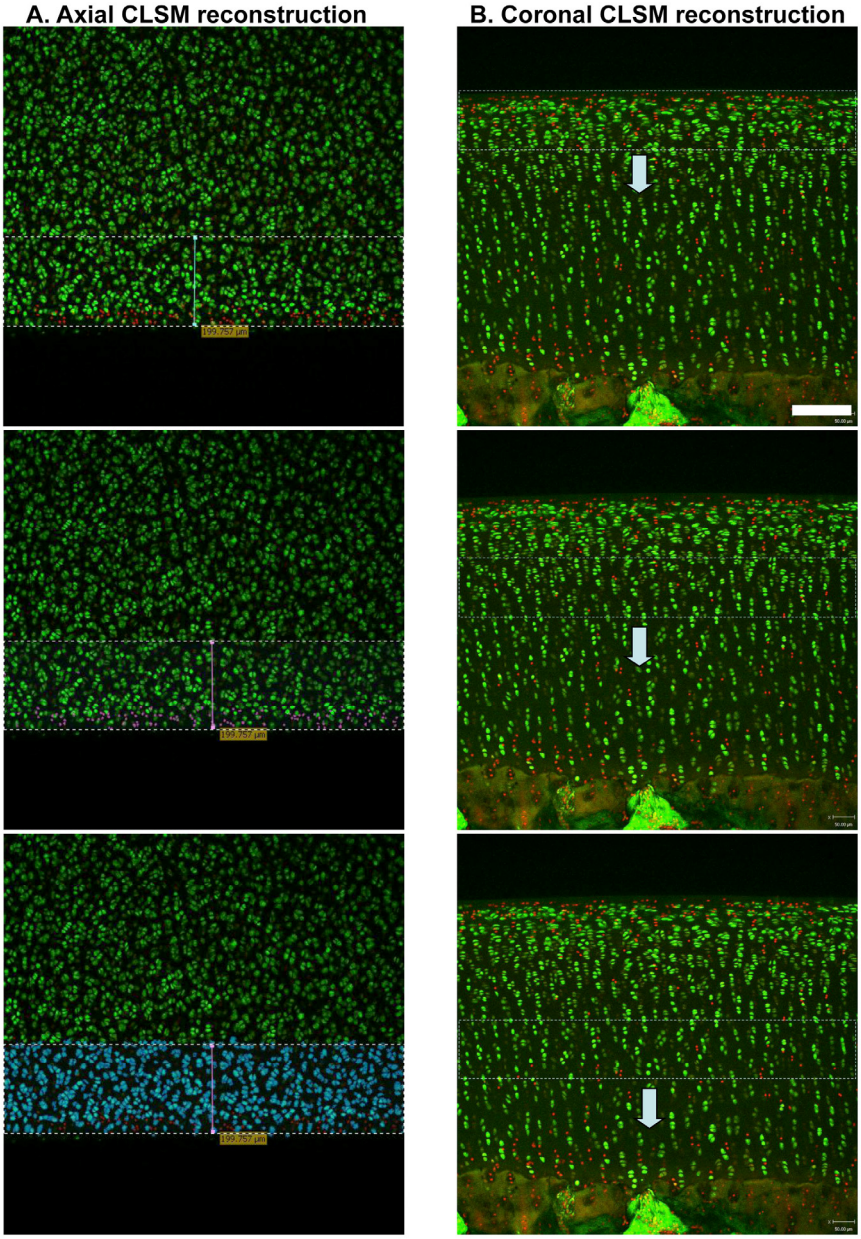


**Figure 2.9: Automated quantitative analyses using CLSM reconstructions**

**Panel A.** An axial CLSM reconstruction of bovine cartilage with a ROI (921 x 200 x 60 µm$^3$, x-y-z axes, respectively) positioned at the cut cartilage edge (top image). Note the PI stained dead cells (middle image, marked in pink) and CMFDA stained live cells (bottom image, marked in blue) have been identified in separate channels using the automated cell counting technique (see section 2.6.2 for details). **Panel B**. A coronal CLSM reconstruction of bovine cartilage with a ROI (921 x 80 x 100 µm$^3$, x-y-z axes, respectively) positioned at 100 µm intervals from the articular surface to quantify percentage cell death as a function of increasing depth from the articular surface.

### 2.6.3 Cartilage cell density

It was important to measure cell density (total number of chondrocytes/$mm^3$ cartilage) for all explants as any variability in cell density over time would affect comparisons of percentage cell death between explants. Such variability in cartilage cellularity between different experimental conditions could occur as a result of potential degradative (Pennock *et al.* 2006a), apoptotic (D'Lima *et al.* 2001a) or proliferative (van Susante *et al.* 2000) processes during culture. In addition, measured values of cell density for the different experimental groups allowed comparisons with reported zone-specific measurements of cell density in bovine and human articular cartilage derived using conventional histological and stereological techniques (Wong *et al.* 1996;Hunziker *et al.* 2002;Stockwell & Meachim 1973). Such comparisons of cell density measurements provided an indication of the reliability, accuracy and precision of the quantitative methodology.

In bovine articular cartilage, cell density was measured within coronal and axial CLSM reconstructions at 2.5 hours and 7 days by dividing the total number of chondrocytes (dead and alive) by the imaged volume of cartilage. In human articular cartilage, cell density within the full thickness of articular cartilage was calculated as the mean cell density in coronal CLSM reconstructions of the superficial, middle and deep zones of articular cartilage for each explant. In human cartilage, cell density within the superficial zone was calculated from the axial CLSM reconstructions of each explant and represented cell density within the uppermost 100μm of articular cartilage i.e. within the region of the superficial zone closest to the articular surface.

## 2.7 STATISTICAL ANALYSES

All statistical analyses were performed using SPSS v. 13.0 (SPSS Inc., Chicago, Illinois, USA). For the experimental methodology, 'N' refers to the number of different animals or patients and 'n', the number of explants for each experiment. The 'N' and 'n' values for each experiment are detailed in the results section of each chapter. If experiments included replicates of explants from the same animal or patient, the values were averaged to obtain a single data point for each animal or patient. Normality of the continuous variables was assessed before statistical analyses using histograms and the Kolmogorov-Smirnov statistic. For non-parametric data, the Wilcoxon signed rank test was used to compare paired observations. For parametric data, the unpaired t-test was used to compare independent observations and the paired t-test was used to compare paired observations; one-way analyses of variance (ANOVA) were conducted to compare trends in observations between the continuous (dependent) variables and categorical (independent) variables. For the data set in section 8.4.2, a two-way ANOVA (between-groups) was conducted to compare percentage cell death in the coronal CLSM reconstructions (continuous dependent variable) in the three zones of articular cartilage (first categorical independent variable with three levels: superficial zone, middle zone and deep zone) between two experimental groups (second categorical variable with two levels). The analyses compared (1) differences in percentage cell death at 2.5 hours ('baseline') and each of the four remaining experimental groups at 7 days ('bone excised', 'bone attached', 'bone excised, healthy bone co-cultured' and 'bone excised, sclerotic bone co-cultured') (2) differences in percentage cell death at 7 days between the experimental group labelled 'bone attached' with each of the

remaining three experimental groups (‘bone excised’, ‘bone excised, healthy bone co-cultured’ and ‘bone excised, sclerotic bone co-cultured’). Levene’s test of equality of error variances was not significant for comparisons between any groups confirming that the variance of the dependent variable across the different groups was equal. The interaction effect between the two main categorical variables was not significant for any of the comparisons and therefore only the main effect for each of the two experimental groups compared is reported. Independent samples t-tests were performed to compare percentage cell death individually in the superficial, middle and deep zones of cartilage at 2.5 hours (baseline) and each of the four remaining experimental groups at 7 days (‘bone excised’, ‘bone attached’, ‘bone excised, healthy bone co-cultured’ and ‘bone excised, sclerotic bone co-cultured’).

All data are presented as means ± standard error for ‘N’ observations. All statistical comparisons were made for ‘N’ observations between experimental groups with $N \geq 3$ for all analyses. The level of significance was set at $p<0.05$.

# CHAPTER 3

# CHONDROCYTE DEATH IN INJURED BOVINE ARTICULAR CARTILAGE - EFFECT OF VARYING CULTURE MEDIUM OSMOLARITY

## 3.1 HYPOTHESES

### Primary hypothesis

*In situ* chondrocyte death in injured articular cartilage is increased by exposure to low medium osmolarity and decreased by exposure to a higher medium osmolarity.

### Secondary hypothesis

Progressive cell death occurs within days in injured articular cartilage, irrespective of medium osmolarity.

## 3.2 CHAPTER SUMMARY

The objective of was to determine whether medium osmolarity affects chondrocyte death in injured bovine articular cartilage. Using CLSM, the spatial distribution of *in situ* chondrocyte death and percentage cell death at the injured cartilage edge were compared as a function of osmolarity and time (2.5 hours vs. 7 days). *In situ* chondrocyte death was mainly localised to the superficial zone of injured articular cartilage for the range of medium osmolarities (0-480 mOsm) at 2.5 hours and 7 days. Compared to the control explants exposed to 340 mOsm DMEM, percentage cell death in the superficial zone was greatest for explants exposed to 0 mOsm (distilled water) and least for explants exposed to 480 mOsm DMEM at 2.5 hours and 7 days ($p<0.05$, paired comparisons versus control). There was no significant change in percentage cell death from 2.5 hours to 7 days for injured explants initially exposed to any of the medium osmolarities ($p>0.05$, paired comparisons at each medium osmolarity). In summary, medium osmolarity significantly affected chondrocyte death in injured articular cartilage. Greatest chondrocyte death occurred at 0 mOsm (distilled water). Conversely, increased medium osmolarity (480 mOsm)

was chondroprotective. The majority of cell death occurred within 2.5 hours, with no further increase over 7 days.

## 3.3 CHAPTER INTRODUCTION

Experimental injury of articular cartilage - for instance, with a scalpel or trephine - is associated with a 'zone' of chondrocyte death and extracellular matrix degradation at the wounded edge (Tew *et al.* 2000;Redman *et al.* 2004). Irrespective of whether the cartilage wound is a result of blunt or sharp injury, extracellular matrix damage in the area of the mechanically injured cartilage is characterised by disruption of the collagen network and loss of proteoglycans (Tew *et al.* 2000;Quinn *et al.* 2001;D'Lima *et al.* 2001a;D'Lima *et al.* 2001b;Redman *et al.* 2004).

Proteoglycans carry fixed negative charges and attract a high concentration of cations (Urban 1994). Since chondrocytes in cartilage are embedded in a matrix containing a high concentration of proteoglycans, it is the local proteoglycan concentrations that dictate the ionic composition and extracellular osmolarity within cartilage via the Gibbs-Donnan equilibrium conditions (Maroudas 1972;Urban *et al.* 1993;Urban 1994). The ionic environment in cartilage is thus different from that of most other mammalian cells, synovial fluid or plasma with a high concentration of cations such as $Na^+$ (250-350 mM), $K^+$ (8-10 mM) and $Ca^{2+}$ (up to 20 mM), a low concentration of anions such as $Cl^-$ (60-90 mM) and a higher extracellular osmolarity (ranging between 350-450 mOsm) than in surrounding tissues (Maroudas 1972). Due to this osmotic pressure difference between cartilage and surrounding tissues, fluid tends to be imbibed by cartilage with the resultant swelling pressure resisted by an intact

collagen network (Maroudas 1976;Urban 1994). Consequently, the net result of extracellular matrix degradation - associated with cartilage injury or degenerative disease states such as osteoarthritis - is cartilage swelling and a decrease in extracellular osmolarity (Maroudas 1976;Maroudas & Venn 1977;Maroudas *et al.* 1985;Jeffrey *et al.* 1995;Farquhar *et al.* 1996;Bank *et al.* 2000).

*In situ* chondrocytes are osmotically sensitive and respond to changes in extracellular osmolarity with reciprocal changes in cell volume (Bush *et al.* 2001b;Bush *et al.* 2005a). The response is mainly controlled by water movement across the plasma membrane due to a differential osmotic pressure gradient (Hoffmann & Dunham 1995). The water transport occurs fairly rapidly with a corresponding change in cell volume within seconds (McGann *et al.* 1988;Bush *et al.* 2001b;Bush *et al.* 2005a). It is however important for chondrocytes to maintain their cell volume to optimise cellular function (Urban *et al.* 1993;Urban 1994). Thus chondrocytes possess regulatory mechanisms based on membrane transporters which serve to restore cell volume to its resting state (Bush *et al.* 2010), although such mechanisms typically take much longer (minutes to hours) than the initial volume response to an alteration in extracellular osmolarity (Bush & Hall 2001a). Further, regulatory cell volume decrease (cell shrinkage after cell swelling from decreased extracellular osmolarity) occurs more readily (Bush *et al.* 2001a) than regulatory cell volume increase (cell swelling during recovery from cell shrinkage) (Kerrigan *et al.* 2006). In a study investigating the osmotic sensitivity of *in situ* bovine chondrocytes with volume-regulatory pathways normally activated by cell shrinkage or swelling blocked by bumetanide/REV5901, there was a rapid (2-3 minutes) change in chondrocyte

volume (quantified by measuring the volume of fluorescent labelled chondrocytes using CLSM). The medium osmolarity in the experiment was varied between 0-530 mOsm (corresponding to an extracellular osmolarity of ~150-600 mOsm) (Bush *et al.* 2001b). This reciprocal relationship between extracellular osmolarity and 'passive' chondrocyte volume also occurs within human articular cartilage (Bush *et al.* 2005a).

Very little is known regarding whether experimental modulation of extracellular osmolarity influences *in situ* chondrocyte responses to mechanical injury. In one previous study, medium (and hence, extracellular) osmolarity was inversely related to the extent of chondrocyte death following an injurious impact (100g weight dropped from a height of 10 cm) as inferred from the release of lactate dehydrogenase (LDH; an intracellular enzyme released following cell injury) into the culture medium (Bush *et al.* 2005b). These data suggested that chondrocytes may be more susceptible to mechanical injury in media of low osmolarity (due to increased cell volume) and protected at a higher osmolarity (due to decreased cell volume). While this was the first reported experiment that studied the relationship between mechanical injury, modulation of extracellular osmolarity and chondrocyte viability (evaluated using a biochemical assay), the spatial and temporal patterns of *in situ* cell death within injured articular cartilage were not characterised as a function of medium osmolarity. The primary hypothesis for this experiment was that *in situ* chondrocyte death in injured articular cartilage is increased by exposure to low medium osmolarity and decreased by exposure to a higher medium osmolarity.

In mechanically injured cartilage, initial chondrocyte death is followed by a secondary, delayed phase of cell death and matrix damage over days (Chen *et al.* 2001;Levin *et al.* 2001;D'Lima *et al.* 2001a). With canine cartilage explants subjected to cyclic indentation impacts of 5 megapascals at 0.3 Hz for 0, 2, 20, and 120 minutes and then kept in culture for 2, 4, 48, and 144 hours, there was a dose-dependent response to the duration of loading and increase in cell death over time (Chen *et al.* 2001). With full thickness cylindrical human cartilage explants (5 mm in diameter) subjected to a 30% strain over 7 days within a custom loading apparatus, the percentage of dead chondrocytes determined using TUNEL labelling demonstrated a progressive increase from 6 hours to 7 days post-injury (D'Lima *et al.* 2001a). The precise mechanisms of this secondary, progressive cell death are still being defined and may involve complex three-way interactions between the chondrocyte, extracellular matrix and soluble mediators (Levin *et al.* 2001;Kuhn *et al.* 2004). This was illustrated in a 'disc-ring' model of canine cartilage where cyclic impacts were applied to a central core of a cartilage disc (Levin *et al.* 2001). The cartilage discs were then cultured over 3, 6 or 21 days either intact or with the core removed after impact (core and unimpacted ring cultured separately). For intact cartilage discs, the extent of cell death was similar in the unimpacted ring and in the impacted core region. However, with the impacted core removed, the extent of cell death in the unimpacted ring was close to control levels, even after 21 days of culture. These findings indicated that the progression of cell death in intact discs may have occurred due to intercellular signalling through the cartilage matrix. The secondary hypothesis for this experiment was that progressive cell death occurs within days in injured articular cartilage, irrespective of medium osmolarity.

The objective was to determine whether altering medium osmolarity affects chondrocyte death in injured bovine articular cartilage. Using CLSM, *in situ* chondrocyte death at the injured cartilage edge has been spatially defined and quantified as a function of medium osmolarity and time (2.5 hours vs. 7 days).

## 3.4 RESULTS

### 3.4.1 Spatial distribution of cell death

Coronal CLSM reconstructions (imaging the full thickness of injured cartilage; N=3, n=12), demonstrated *in situ* chondrocyte death mainly localised to the superficial zone - with sparing of the middle and deeper zones - for explants exposed to the range of medium osmolarities (0-480 mOsm), at 2.5 hours and 7 days (representative CLSM reconstructions for 0 mOsm and 340 mOsm shown in **Figure 3.1**).

Axial CLSM reconstructions (imaging the superficial zone 'top down' from the articular surface) demonstrated a decrease in the extent of *in situ* chondrocyte death at the injured cartilage edge with increasing medium osmolarity at 2.5 hours and 7 days. Additionally, *in situ* chondrocytes within the adjacent uninjured region of the articular surface were unaffected by exposure to the range of medium osmolarities (0-480 mOsm) with no visible cell death in this region (**Figure 3.2**).

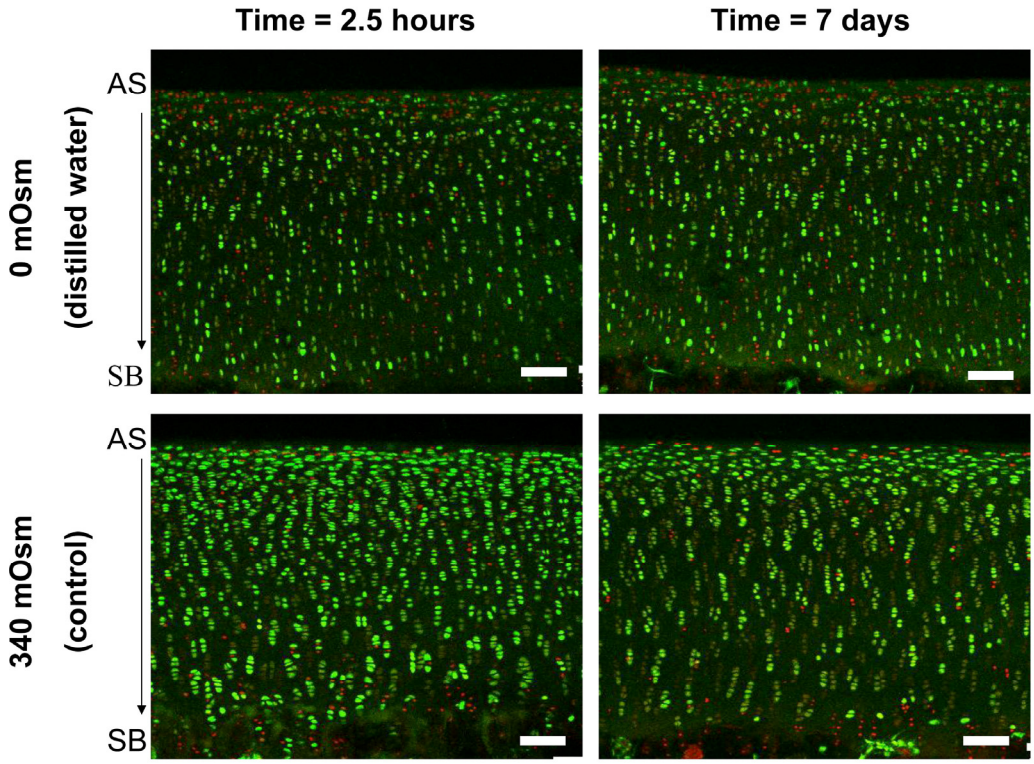


**Figure 3.1: Representative coronal CLSM reconstructions showing *in situ* chondrocyte death localised in the superficial zone at 2.5 hours and 7 days for explants exposed to distilled water (0 mOsm) or DMEM (control, 340 mOsm)**

Panel shows coronal CLSM reconstructions of the scalpel cut surface (imaging all zones within the full thickness of injured cartilage) with *in situ* chondrocyte death predominantly localised to the superficial zone for explants exposed to 0 mOsm (distilled water) and 340 mOsm DMEM (control) at 2.5 hours and 7 days. Within the middle and deep zones, there was some cell death at the cut surface (from direct scalpel trauma) but cells further away remained viable. In the superficial zone however, there was more extensive cell death (AS=articular surface; SB= Subchondral bone, white bar = 100μm).

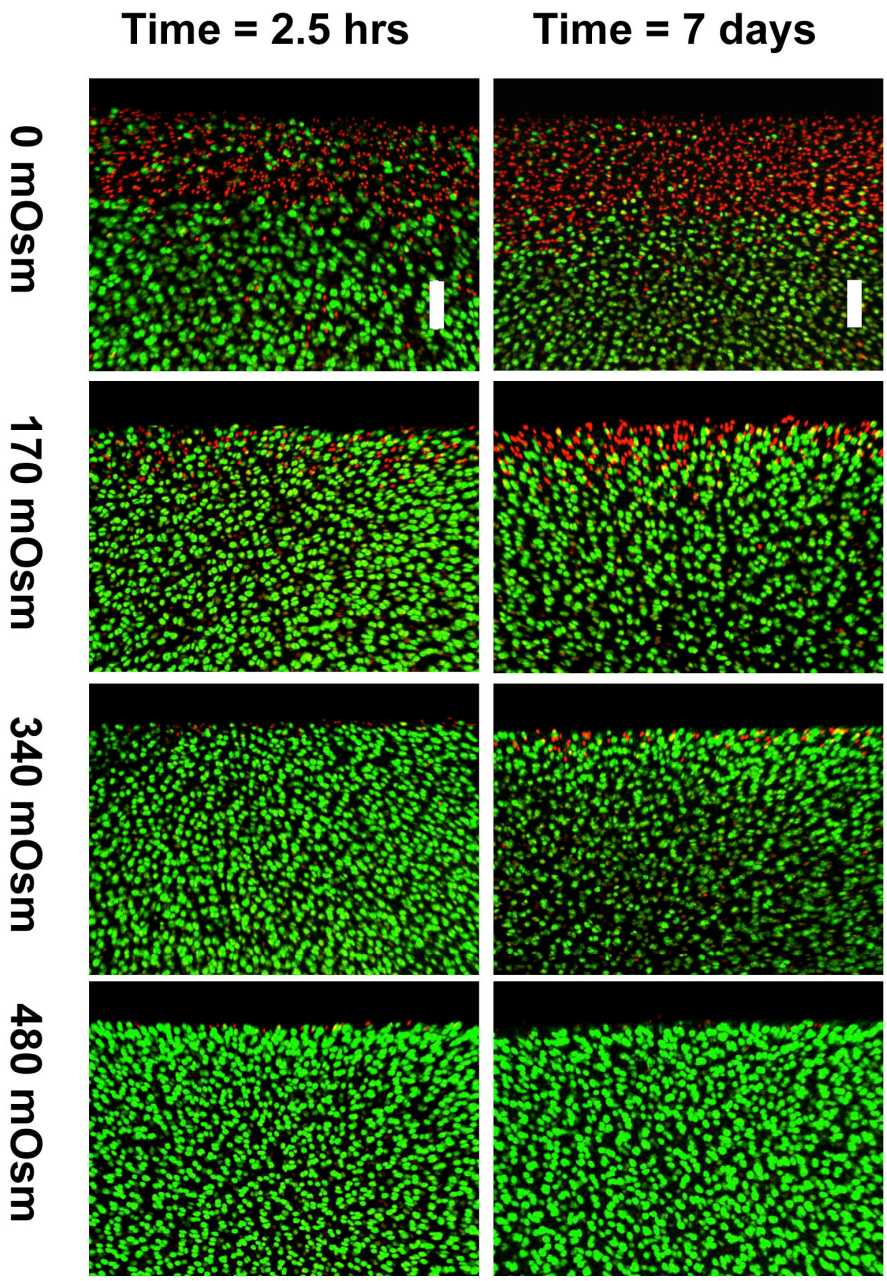


**Figure 3.2: Axial CLSM reconstructions showing *in situ* chondrocyte death at the injured cartilage edge as a function of medium osmolarity and time**

Panels show axial CLSM reconstructions imaging the cut edge of cartilage 'top down' from the articular surface with a band of *in situ* chondrocyte death in the superficial zone from mechanical (scalpel) injury at the cartilage edge. The extent of chondrocyte death at the injured edge decreases with increasing medium osmolarity at both 2.5 hours and 7 days. Note the 'chondroprotective' effect of high (480 mOsm) medium osmolarity compared to the extensive chondrocyte death at low (0 mOsm) medium osmolarity (white bar = 100μm).

### 3.4.2 Percentage cell death

As *in situ* chondrocyte death localised to the articular surface, percentage cell death was quantified from axial CLSM reconstructions that exclusively imaged the superficial zone. Percentage cell death was significantly higher for explants exposed to 0 mOsm (distilled water) compared to explants exposed to 340 mOsm DMEM (controls) at 2.5 hours (N=6, n=12, p=0.04, Wilcoxon signed rank test) and 7 days (N=6, n=12, p=0.03, **Figure 3.3**). However, percentage cell death was significantly lower for explants exposed to 480 mOsm DMEM compared to the controls at 2.5 hours (N=6, n=12, p=0.03) and 7 days (N=6, n=12, p=0.046). There was no significant difference in the percentage cell death for explants exposed to 170 mOsm DMEM compared to the controls at 2.5 hours (N=6, n=12, p=0.7) and 7 days (N=6, n=12, p=0.2). The change in percentage cell death from 2.5 hours to 7 days was not statistically significant for explants exposed to all four medium osmolarities (**Table 3.1**). These data suggest that the majority of the chondrocyte death occurred within 2.5 hours of cartilage injury and there was no further progression of cell death over 7 days.

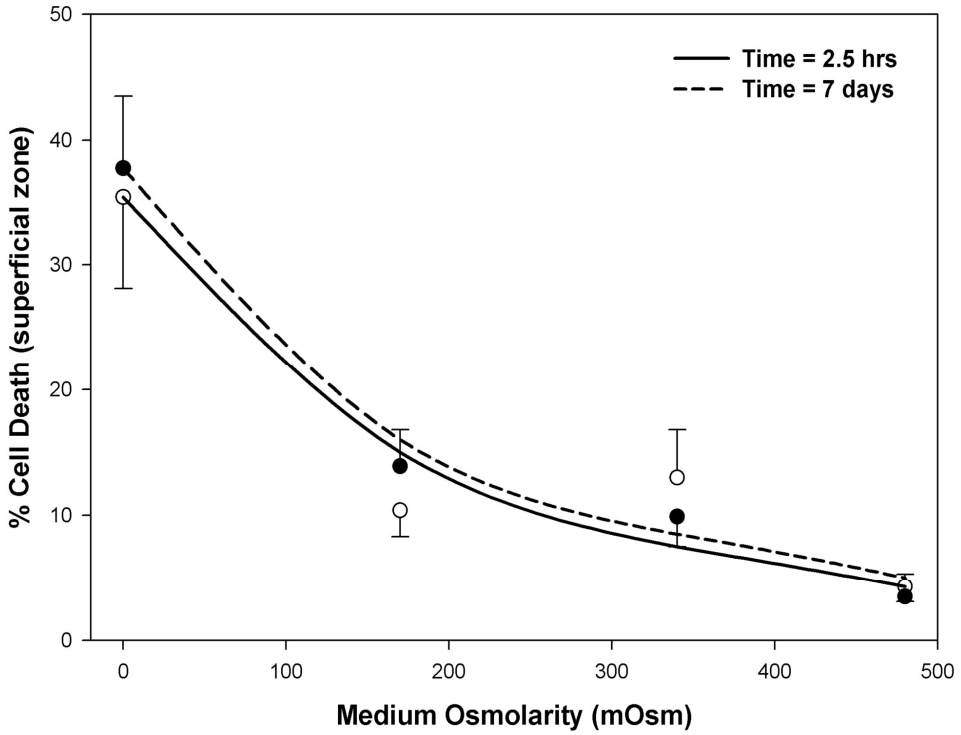


**Figure 3.3: Pooled data for percentage cell death as a function of medium (DMEM) osmolarity and time**

Percentage cell death decreased with increasing medium (DMEM) osmolarity at 2.5 hours and 7 days. However, there was no significant increase in cell death from 2.5 hours to 7 days for any of the four medium osmolarities (see section 3.4.2 entitled 'Percentage cell death' and Table 3.1 for statistical data).

| Medium Osmolarity (mOsm) | No. dead : live cells at 2.5 hrs | No. dead : live cells at 7 days | % change (2.5 hrs to 7 days) | p-value* |
|---|---|---|---|---|
| **0** | 341 ± 47 : 728 ± 200 | 275 ± 50 : 458 ± 61 | 2.2 | 0.3 |
| **170** | 83 ± 13 : 749 ± 79 | 118 ± 17 : 805 ± 113 | 3.5 | 0.07 |
| **340** # | 79 ± 28 : 587 ± 118 | 83 ± 23 : 737 ± 43 | -3.1 | 0.2 |
| **480** | 28 ± 5 : 664 ± 78 | 31 ± 5 : 840 ± 68 | -0.8 | 0.6 |

**TABLE 3.1: Change in percentage cell death as a function of time**

Table shows dead and live cell counts within a region of interest measuring 921μm (x axis) x 200μm (y axis) x 60μm (z axis) positioned at the cut cartilage edge on axial CLSM reconstructions. The change in percentage cell death from 2.5 hours to 7 days was not statistically significant for explants exposed to all four medium osmolarities (all data shown as means ± standard error, *Wilcoxon signed rank test for paired observations, #standard culture medium and control).

### 3.4.3 Cartilage cell density

There was no change in cartilage cell density in the superficial zone (total number of chondrocytes (dead and alive)/$mm^3$ calculated from axial CLSM reconstructions) from 2.5 hours to 7 days for explants exposed to all four medium osmolarities (**Table 3.2**) indicating that there was no change in cartilage cellularity between different experimental conditions over 7 days in culture that could otherwise have affected comparisons of percentage cell death between explants. There was also no significant difference in cartilage cell density between explants exposed to the four different medium osmolarities at 2.5 hours and 7 days ($p>0.05$ for paired comparisons,

Wilcoxon signed rank tests versus controls) indicating that explants in different experimental groups were obtained from homogenous areas of cartilage with similar cell density.

| **Osmolarity (mOsm)** | **Cell density at 2.5 hrs (No. of cells/ $mm^3x10^3$)** | **Cell density at 7 days (No. of cells/ $mm^3x10^3$)** | **p-value*** |
|---|---|---|---|
| **0** | 96.7 ± 17.6 | 66.3 ± 6.1 | 0.2 |
| **170** | 75.2 ± 7.3 | 83.6 ± 10.4 | 0.5 |
| **340** # | 60.2 ± 11.2 | 74.2 ± 4.5 | 0.5 |
| **480** | 62.6 ± 6.9 | 78.8 ± 6.4 | 0.2 |

**TABLE 3.2: Cartilage cell density at 2.5 hours and 7 days in the superficial zone**

Cartilage cell density in the superficial zone was calculated from axial CLSM reconstructions. There is no change in cartilage cell density from 2.5 hours to 7 days for explants exposed to all four medium osmolarities (all data shown as means ± standard error, *Wilcoxon signed rank test for paired observations, # standard culture medium and control).

## 3.5 CHAPTER DISCUSSION

Two key findings can be surmised from the experiments in which bovine articular cartilage was injured with a single scalpel cut. First, medium osmolarity significantly influenced superficial zone chondrocyte death at the injured edge with the greatest cell death occurring at 0 mOsm (distilled water) - conversely, a high medium osmolarity (480 mOsm) was chondroprotective. Second, in this bovine model the majority of *in situ* chondrocyte death at the injured edge occurred within 2.5 hours, with no further increase in cell death over 7 days, irrespective of medium osmolarity.

The spatial distribution of *in situ* chondrocyte death in injured articular cartilage suggested localisation to the superficial zone, and compares with similar observations in cartilage subjected to impact and cyclical trauma (Jeffrey *et al.* 1995;Quinn *et al.* 2001;Lewis *et al.* 2003;Chen *et al.* 2003;Bush *et al.* 2005b). The integrity of the superficial zone is crucial in maintaining the biological and mechanical properties of articular cartilage and preventing progressive cartilage degeneration (Maroudas 1976;Hunziker 2002;Hunziker *et al.* 2002). Although reasons for the localisation of chondrocyte death (from mechanical injury) to the superficial zone are not clear, it is hypothesised that the heterogeneous spatial microstructure of articular cartilage may be implicated for several reasons. Firstly, three-dimensional models of articular cartilage describe chondrocytes embedded within leaf-like arcades of collagen fibres orientated horizontal and parallel to the articular surface in the superficial zone, arching through the middle zone to become perpendicular in the deep zone of articular cartilage (Jeffery *et al.* 1991;Hunziker *et al.* 2002;Hughes *et al.* 2005). Due to this arrangement of the cells and matrix, the compressive stiffness of the superficial zone is significantly less than that of the middle and deep zones (Guilak *et al.* 1995). Any mechanical stress therefore results in greater matrix compaction and tissue strain in the superficial zone compared to deeper zones of articular cartilage (Guilak *et al.* 1995;Milentijevic, Helfet, & Torzilli 2003). Consequently, in response to mechanical stress there is greater cell deformation and cellular disruption in the superficial zone. Secondly, compacted and horizontally orientated chondrocytes within the superficial zone, may be more susceptible to an axially directed mechanical force (Jeffery *et al.* 1991;Hunziker *et al.* 2002;Hughes *et al.* 2005). Within the middle and deep zones of cartilage where

less compacted chondrocytes are orientated more vertically (Hunziker *et al.* 2002;Hughes *et al.* 2005), the force vector may pass in a plane parallel to the orientation of the cells and therefore cause less damage. Finally, the superficial zone has the highest permeability and water content (Maroudas, Muir, & Wingham 1969;Brocklehurst *et al.* 1984) with *in situ* chondrocytes that are more osmotically sensitive (swelling more for a given change in osmolarity) compared to cells located deeper in cartilage (Bush *et al.* 2001b). As the volume of chondrocytes may be an important determinant of the cartilage response to mechanical injury (Bush *et al.* 2005b), the effects of varying medium osmolarity (and hence, chondrocyte volume; see discussion below) may have been most marked within the superficial zone.

These data support the primary hypothesis that *in situ* chondrocyte death in injured articular cartilage is increased by exposure to low medium osmolarity and decreased by exposure to a higher medium osmolarity. Altering medium osmolarity results in rapid water movement across the chondrocyte plasma membrane with corresponding volume changes occurring within seconds (McGann *et al.* 1988;Bush *et al.* 2001b;Bush *et al.* 2005a). It is possible that at low medium osmolarity, swollen chondrocytes were more likely to be directly injured from the scalpel cut by virtue of their larger size, while at a higher medium osmolarity, smaller, shrunken chondrocytes may have been protected from the mechanical trauma (Bush *et al.* 2001b;Bush *et al.* 2005b). However, the spatial pattern of *in situ* chondrocyte death as a function of medium osmolarity (i.e. a sequential decrease in the width of the zone of cell death) suggests that the osmotic responses of chondrocytes may have been exacerbated by the damage to the extracellular matrix from the scalpel injury.

Disruption of the collagen network releases interlocked stresses within articular cartilage (Fry 1974), with 'debonding' of the layered collagen arrangement (Clark & Simonian 1997). A damaged collagen network is unable to resist the intrinsic, proteoglycan-mediated cartilage swelling and becomes more permeable to fluid (Maroudas 1976;Maroudas *et al.* 1977;Maroudas *et al.* 1985;Torzilli *et al.* 1999;Bank *et al.* 2000). It is hypothesised that at low medium osmolarity, injured cartilage rapidly imbibes fluid at the damaged surface reducing extracellular osmolarity and consequently, swelling *in situ* chondrocytes (Bush *et al.* 2001b) which, in the absence of any resistance from an intact collagen network, swell even further with cell lysis. Conversely, in the presence of a higher medium osmolarity, the fluid imbibition onto the injured surface of articular cartilage may be prevented avoiding swelling-induced lysis. Cell death localised around impact induced cracks in articular cartilage but not in impacted areas without cracks (Lewis *et al.* 2003), supports the view that disruption of the extracellular matrix may be linked to chondrocyte death following mechanical injury.

These data do not support the secondary hypothesis - progressive cell death was not observed in injured cartilage despite culture of explants over 7 days. This contrasts with findings of delayed, secondary cell death that occurred over a period of 6 hours to 7 days after initial cartilage injury with a trephine (Tew *et al.* 2000;Redman *et al.* 2004) or blunt impact (Chen *et al.* 2001;Levin *et al.* 2001;D'Lima *et al.* 2001a;Borazjani *et al.* 2006). The total cell counts and cell density measurements in the superficial zone for each of the four experimental groups were similar (**Table 3.1 and 3.2**) and comparable to reported values using conventional histological and

stereological techniques (Wong *et al.* 1996;Stockwell & Meachim 1973). Further, there was no change in cell density from 2.5 hours to 7 days under any of the experimental conditions (**Table 3.2**), confirming that there were no net alterations in cartilage cellularity due to degradative (Pennock *et al.* 2006a), apoptotic (D'Lima *et al.* 2001a) or proliferative (Tew *et al.* 2000;van Susante *et al.* 2000;Redman *et al.* 2004) processes during culture that would have affected comparisons of percentage cell death for the different experiment groups.

It is noteworthy that in studies that have found a progression of chondrocyte death over time (days) after mechanical injury (Tew *et al.* 2000;Chen *et al.* 2001;Levin *et al.* 2001;D'Lima *et al.* 2001a;Redman *et al.* 2004;Borazjani *et al.* 2006), cartilage (animal and human) was excised from subchondral bone immediately before or after the mechanical insult. Intriguingly, when articular cartilage has been left attached to subchondral bone, no progression of chondrocyte death occurred after impact trauma (Lewis *et al.* 2003;Bush *et al.* 2005b), injurious compression (Loening *et al.* 2000) or cyclical load (Chen *et al.* 2003). Reasons for this association between progressive chondrocyte death and subchondral bone remain to be elucidated, but the reduced physical disruption of the extracellular matrix from mechanical trauma when left attached to subchondral bone (Jeffrey *et al.* 1995;Krueger *et al.* 2003), cell-matrix interactions from loss of anchorage to subchondral bone (Buckwalter *et al.* 1998b;Kuhn *et al.* 2004) and cartilage swelling following excision from sub-chondral bone (Maroudas 1976;Grushko, Schneiderman, & Maroudas 1989;Huntley *et al.* 2005c), may all be implicated. It is possible that the extent of chondrocyte death associated with a single scalpel cut in this experiment was much less compared to

that resulting from impact or blunt trauma (Redman *et al.* 2004;Huntley *et al.* 2005a) and may have suppressed chondrocyte-extracellular matrix signalling responses known to propagate cell death (Levin *et al.* 2001;Kuhn *et al.* 2004). The potential chondroprotective effect of cartilage attachment to subchondral bone is considered further in Chapters 7 and 8.

In conclusion, medium osmolarity significantly influences superficial zone chondrocyte death in injured articular cartilage. Greatest chondrocyte death occurred at 0 mOsm (distilled water). Conversely, a raised medium osmolarity (480 mOsm) was chondroprotective. The majority of *in situ* chondrocyte death occurred within 2.5 hours, with no further increase over 7 days.

# CHAPTER 4

# CHONDROCYTE DEATH IN INJURED BOVINE ARTICULAR CARTILAGE - EFFECT OF VARYING MEDIUM CALCIUM CONCENTRATION

## 4.1 HYPOTHESES

### Primary Hypothesis

Exposure of articular cartilage to calcium-free media (~0 mM) decreases *in situ* chondrocyte death following mechanical injury (within hours).

### Secondary Hypothesis

Culture of injured articular cartilage in calcium-rich media (2-20 mM) increases *in situ* chondrocyte death (within days).

## 4.2 CHAPTER SUMMARY

The objective was to determine whether medium calcium concentration affects chondrocyte death in injured bovine articular cartilage. Using CLSM, the spatial distribution of *in situ* chondrocyte death and percentage cell death at the injured cartilage edge were compared as a function of medium calcium concentration and time (2.5 hours vs. 7 days). Exposure of articular cartilage to calcium-free media (~0 mM) significantly reduced superficial zone chondrocyte death at 2.5 hours after mechanical injury compared with exposure to calcium-rich media (2-20 mM, ANOVA, $p=0.002$). In calcium-rich media, although the extent of chondrocyte death increased with increasing medium calcium concentration, cell death remained localised to the superficial zone of articular cartilage over 7 days (ANOVA, $p<0.05$). However, in calcium-free media, there was an increase in chondrocyte death within deeper zones of injured articular cartilage over 7 days.

## 4.3 CHAPTER INTRODUCTION

Calcium plays an important role in the responses of articular chondrocytes to external mechanical stimuli (Guilak *et al.* 1999;Roberts *et al.* 2001;Browning *et al.* 2004). In isolated bovine articular chondrocytes, short applications of high hydrostatic pressures led to a significant increase in intracellular calcium concentrations (Browning *et al.* 2004). Controlled deformation of isolated bovine articular chondrocytes with the edge of a glass micropipette resulted in an immediate and transient increase in intracellular calcium ion concentrations (Guilak *et al.* 1999). Further, the initiation of intracellular calcium ion waves was abolished by removing calcium from the extracellular media or by adding inhibitors of mechanosensitive ion channels to the media such as gadolinium or amiloride (Guilak *et al.* 1999;Browning *et al.* 2004). In a study using bovine articular chondrocytes embedded in 4% agarose constructs with physiological mechanical compressive strain applied after 1 and 3 days in culture, there was a significant increase in intracellular calcium ion concentration (measured using CLSM) in strained constructs compared with unstrained controls at both time points. In the strained constructs, treatment with either gadolinium or EGTA significantly reduced the positive calcium ion response compared with untreated controls (Roberts *et al.* 2001). These findings indicated that a transient increase in intracellular calcium concentration may be one of the earliest events involved in the response of chondrocytes to mechanical stress and that deformation-induced calcium ion waves were initiated through mechanosensitive ion channels (Guilak *et al.* 1999).

Despite the established role of calcium signalling in regulating pathways of mechanotransduction, little is known regarding the influence of extracellular calcium on *in situ* chondrocyte death following mechanical injury. In equine cartilage subjected to a single impact load (500 g weight dropped from a height of 50 mm), a reduction in extracellular calcium (by chelating calcium from the culture media using EGTA), significantly decreased impact induced chondrocyte death after 48 hours in culture (Huser & Davies 2007). In the study, chondrocyte death was quantified in the superficial zone of cartilage from coronal histological sections using the TUNEL method, but the spatial distribution of *in situ* chondrocyte death within the full thickness of cartilage was not investigated as a function of medium calcium concentration or time. Nevertheless, the chondroprotection conferred by decreasing the calcium ion concentration in the extracellular media is relevant to the scalpel-induced mechanical injury investigated in this thesis. The primary hypothesis was that exposure of articular cartilage to calcium-free media (~0 mM) decreases *in situ* chondrocyte death following mechanical injury (within hours).

While a decrease in extracellular calcium concentration may reduce chondrocyte death following mechanical injury (Mansfield *et al.* 2003;Huser *et al.* 2007), the relationship between an increase in extracellular calcium concentration (or 'calcium overload') and progressive chondrocyte death after mechanical injury has not been investigated. In degenerative joint disorders such as osteoarthritis, there is an increase in extracellular calcium concentrations (Rizzo *et al.* 1995) and in other cell types, such as neurones, a link has been established between calcium overload, increased programmed cell death and the development of neurodegenerative

disorders (Mattson & Chan 2003;Mattson 2006). The secondary hypothesis for the experiments was that culture of injured articular cartilage in calcium-rich media (2-20 mM) increases *in situ* chondrocyte death (within days).

The objective was to determine whether medium calcium concentration affects chondrocyte death in injured bovine articular cartilage. Using CLSM, *in situ* chondrocyte death at the injured cartilage edge has been spatially defined and quantified as a function of medium calcium concentration and time (2.5 hours vs. 7 days).

## 4.4 RESULTS

### 4.4.1 Spatial distribution of cell death

Coronal CLSM reconstructions of the full thickness of scalpel injured cartilage demonstrated *in situ* chondrocyte death localised mainly within the superficial zone, with relative sparing of the middle and deeper zones of articular cartilage for explants exposed to calcium-free (0 mM) and calcium-rich (2-20 mM) media at 2.5 hours. Although, chondrocyte death remained localised to the superficial zone for explants cultured in calcium-rich media over 7 days, cell death progressed to involve the middle and deep zones of cartilage for explants cultured over 7 days in calcium-free media (**Figure 4.1,** see section 4.4.2 for quantitative data).

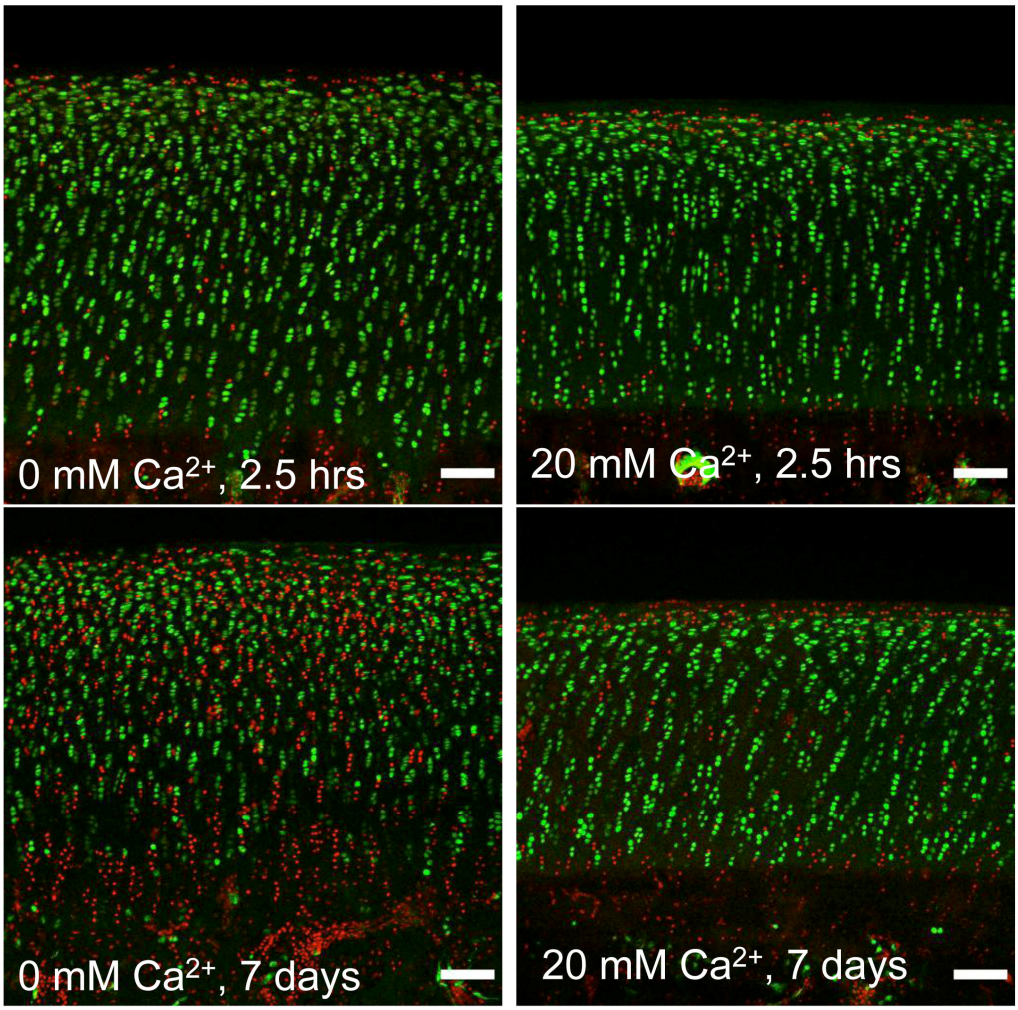


**Figure 4.1: Coronal CLSM reconstructions show the increase in cell death within deeper zones of articular cartilage in calcium-free media over 7 days**

At 2.5 hours, chondrocyte death was localised mainly within the superficial zone, with relative sparing of the middle and deeper zones of articular cartilage for explants exposed to calcium-free media (top left image labelled '0mM $Ca^{2+}$, 2.5 hrs') and calcium-rich media (top right image labelled '20mM $Ca^{2+}$, 2.5 hrs'). However, there was a progression of cell death for explants cultured over 7 days in calcium-free media (bottom left image labelled '0mM $Ca^{2+}$, 7 days') with involvement of the middle and deep zones of cartilage. In calcium-rich media (bottom right image labelled '20mM $Ca^{2+}$, 7 days'), chondrocyte death remained localised to the superficial zone over 7 days. (PI stains the nuclei of dead cells red; CMFDA stains the cytoplasm of live cells green, white bar = 100 μm).

Axial CLSM reconstructions of the superficial zone at the articular surface indicated a ‘band’ of cell death at the scalpel injured edge at 2.5 hours and 7 days for the range of medium calcium concentrations (0-20 mM), although the extent of the band of cell death was least for explants exposed to calcium-free (0 mM) media and greatest for explants exposed to media with the highest calcium concentration (20 mM, **Figure 4.2,** see section 4.4.2 for quantitative data). There was no increase in cell death within the adjacent uninjured region of the articular surface at 2.5 hours or 7 days, irrespective of the medium calcium concentration.

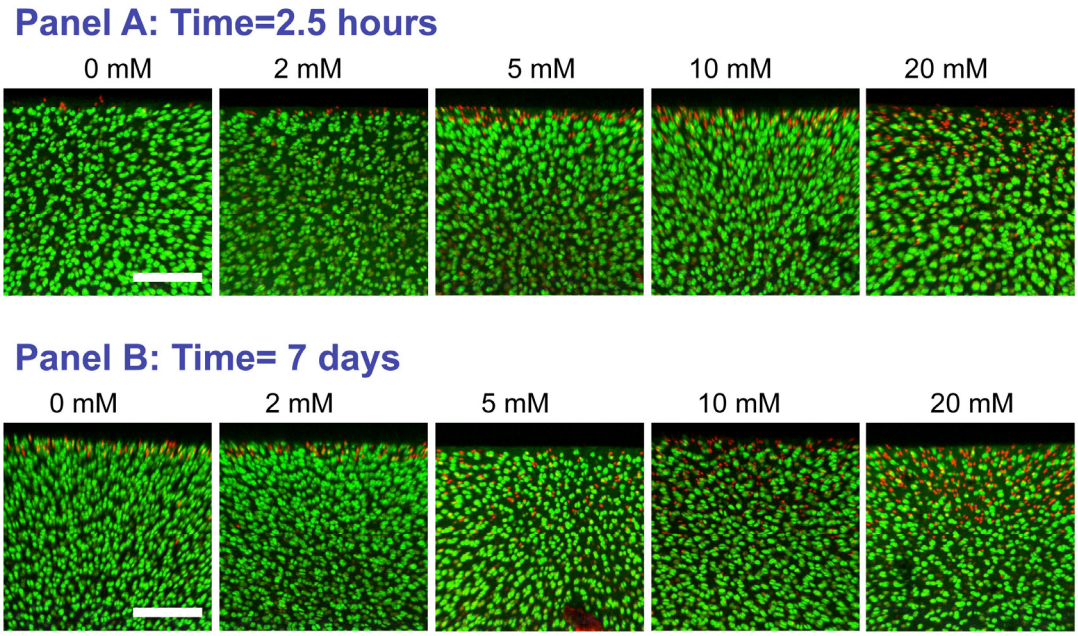


**Figure 4.2: Axial CLSM reconstructions show the increase in chondrocyte death in the superficial zone with increasing medium calcium concentration**

Note the 'band' of cell death from the scalpel injury at the cartilage edge at 2.5 hours and 7 days for the range of medium calcium concentrations (0-20 mM). The extent of cell death at the injured cartilage edge increased with exposure of explants to increasing medium calcium concentration at 2.5 hours (Panel A) and 7 days (Panel B). There was chondroprotection for explants exposed to calcium-free media (0 mM) compared with the more extensive cell death for explants exposed to calcium-rich media (2-20 mM). *In situ* chondrocytes within the adjacent uninjured region of the articular cartilage were viable at 2.5 hours and 7 days irrespective of the medium calcium concentration (white bar = 100 μm).

### 4.4.2 Percentage cell death

Percentage cell death quantified from coronal CLSM reconstructions ($PCD_{FT}$) - imaging the full thickness of articular cartilage - confirmed cell death localised within the first 100 μm from the articular surface for explants exposed to media containing 0, 2, 5, 10 and 20 mM calcium at 2.5 hours (N=6, n=30, one-way

ANOVA for depth from articular surface, $p<0.05$ for all comparisons, **Figure 4.3A**). $PCD_{FT}$ at 7 days indicated that cell death remained localised within the first 100 μm from the articular surface for explants exposed to calcium-rich media (N=6, n=24, 2-20 mM, one-way ANOVA for depth from articular surface, $p<0.05$ for all comparisons, **Figure 4.3B**). However, for explants cultured over 7 days in calcium-free media (0 mM), chondrocyte death did not remain localised within the first 100 μm from the articular surface as indicated by relative increase in $PCD_{FT}$ at depths greater than 100 μm from the articular surface (N=6, n=6, one-way ANOVA for depth from articular surface, $p=0.5$, **Figure 4.3B**).

Percentage cell death quantified from axial CLSM reconstructions (PCDsz) - imaging the band of *in situ* chondrocyte death in the superficial zone - was lowest for explants exposed to calcium-free media and increased significantly with increasing medium calcium concentration at 2.5 hours (N=6, n=30, one-way ANOVA, $p=0.002$, **Figure 4.4**).

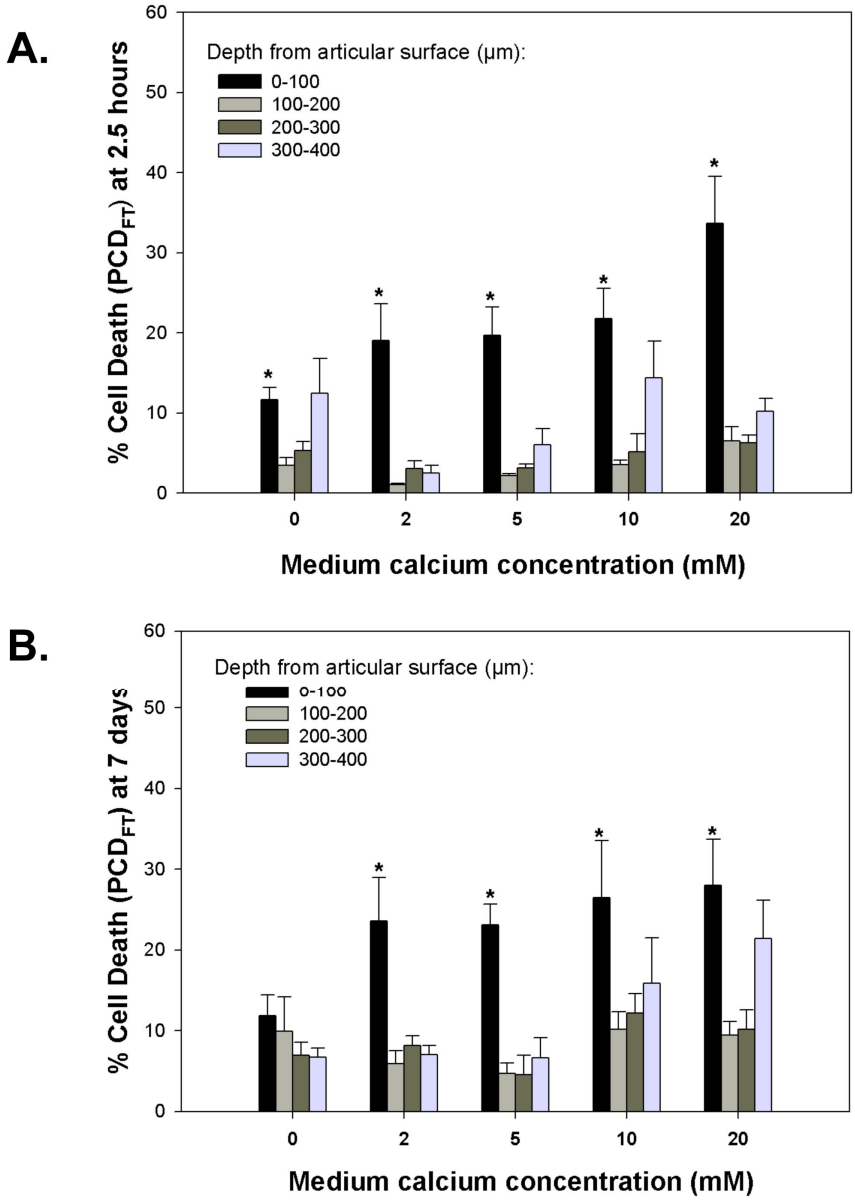


**Figure 4.3: Pooled data for $PCD_{FT}$ as a function of medium calcium concentration and time (2.5 hours and 7 days)**

**A.** $PCD_{FT}$ at 2.5 hours: There is localisation of chondrocyte death to the first 100 µm from the articular surface for the range (0-20 mM) of medium calcium concentrations.

**B.** $PCD_{FT}$ at 7 days: There is localisation of chondrocyte death to the first 100 µm from the articular surface for explants exposed to calcium-rich media (2-20 mM). However, for explants cultured in calcium-free media (0 mM), there was an increase in chondrocyte death at depths greater than 100 µm (* = one-way ANOVA for depth from articular surface, $p<0.05$).

Paired comparisons indicated that PCDsz for explants exposed to calcium-free media (0 mM, PCDsz = 6.1%) was significantly lower than the PCDsz for explants exposed to calcium-rich media (2, 5, 10 and 20 mM, PCDsz = 8.2%, 14.4%, 14.7% and 27.9% respectively, $p<0.05$ for all comparisons). The trend was similar at 7 days with a significant increase in PCDsz with increasing medium calcium concentration (N=6, n=30, one-way ANOVA, p=0.006, **Figure 4.4**). There was no increase in PCDsz from 2.5 hours to 7 days for explants exposed to the range of medium calcium concentrations (N=6, n=60, 0-20 mM, two-way ANOVA, p=0.6).

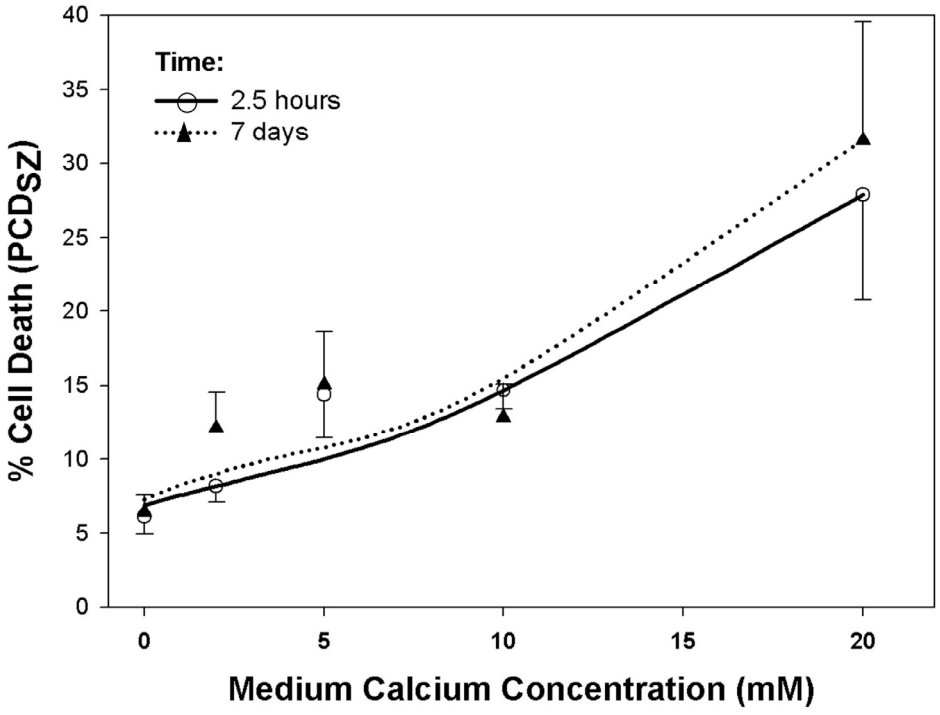


**Figure 4.4: Pooled data for $PCD_{SZ}$ as a function of medium calcium concentration and time (2.5 hours and 7 days)**

PCDsz was lowest for explants exposed to calcium-free media (0 mM) and increased progressively in calcium-rich media (2-20 mM), at 2.5 hours (one-way ANOVA, p=0.002) and 7 days (p=0.006). There was no significant increase in PCDsz from 2.5 hours to 7 days at any medium calcium concentration (two-way ANOVA, p=0.6).

### 4.4.3 Cartilage cell density

There was no change in cell density from 2.5 hours to 7 days within coronal and axial CLSM reconstructions for explants exposed to the range of medium calcium concentrations (unpaired t-tests, $p>0.05$ for all comparisons, **Tables 4.1 and 4.2**).

| **Calcium concentration (mM)** | **Mean cartilage cell density (No. of cells/ $mm^3x10^3$)** | | |
|---|---|---|---|
| | ***2.5 hours*** | ***7 days*** | ***p value*** |
| **0** | 31.4 ± 1.3 | 38.2 ± 2.3 | 0.4 |
| **2** | 32.6 ± 1.5 | 34.5 ± 1.7 | 0.1 |
| **5** | 35.4 ± 1.4 | 39.1 ± 1.6 | 0.1 |
| **10** | 35.0 ± 1.6 | 39.2 ± 1.7 | 0.6 |
| **20** | 38.0 ± 0.7 | 36.9 ± 2.2 | 0.5 |

**Table 4.1: Mean cartilage cell density at 2.5 hours and 7 days**

The mean cartilage cell density was calculated from coronal CLSM reconstructions as the mean cell density to a depth of 400μm from the articular surface, representing >90% of cells within the full thickness of cartilage. There was no change in the measured values of cell density from 2.5 hours to 7 days for the range of medium calcium concentrations (0-20 mM, N=6, n=30, unpaired t-test, $p>0.05$ for all comparisons, all data shown as mean ± standard error).

| **Calcium concentration (mM)** | **Cell density in the superficial zone (No. of cells/ $mm^3x10^3$)** | | |
|---|---|---|---|
| | ***2.5 hours*** | ***7 days*** | ***p value*** |
| **0** | 53.9 ± 1.9 | 56.4 ± 2.8 | 0.5 |
| **2** | 56.5 ± 2.1 | 51.7 ± 5.3 | 0.4 |
| **5** | 65.1 ± 2.6 | 59.0 ± 1.6 | 0.1 |
| **10** | 55.0 ± 3.2 | 54.1 ± 3.0 | 0.8 |
| **20** | 62.8 ± 5.1 | 64.5 ± 2.7 | 0.8 |

**Table 4.2: Cartilage cell density at 2.5 hours and 7 days in the superficial zone**

Cartilage cell density in the superficial zone was calculated from axial CLSM reconstructions. There was no change in the measured values of cell density from 2.5 hours to 7 days for the range of medium calcium concentrations (0-20 mM, N=6, n=30, unpaired t-test, $p>0.05$ for all comparisons, all data shown as mean ± standard error).

## 4.5 CHAPTER DISCUSSION

Two key findings can be surmised from the experiment. First, exposure of articular cartilage to calcium-free media (0 mM) significantly decreased superficial zone chondrocyte death after mechanical injury (within hours) compared with exposure to calcium-rich media (2-20 mM). Second, in calcium-rich media, chondrocyte death increased with increasing medium calcium concentration but remained localised to the superficial zone of articular cartilage over 7 days. However, in calcium-free media, there was an increase in chondrocyte death within deeper zones of injured articular cartilage over 7 days.

Measured values of cell density were comparable to zone-specific measurements in bovine cartilage derived using histological and stereological quantitative techniques (Wong *et al.* 1996;Stockwell & Meachim 1973). Cell density did not change from 2.5 hours to 7 days for explants cultured in either calcium-free or calcium-rich media, indicating that there were no net alterations in cartilage cellularity due to degradative (Pennock *et al.* 2006a), apoptotic (D'Lima *et al.* 2001a) or proliferative (van Susante *et al.* 2000) processes that would have affected subsequent comparisons of percentage cell death between the experimental groups. The spatial distribution of *in situ* chondrocyte death within injured articular cartilage suggested localisation to the superficial zone (with the exception of explants cultured in calcium-free media over 7 days), and compares with similar observations in cartilage subjected to impact and cyclical trauma (Jeffrey *et al.* 1995;Quinn *et al.* 2001;Lewis *et al.* 2003;Chen *et al.* 2003;Bush *et al.* 2005b) as well as with findings reported in Chapter 3.

These data support the primary hypothesis that exposure of articular cartilage to calcium-free media decreases *in situ* chondrocyte death following mechanical injury (within hours) and compares with similar findings in a single-impact model of cartilage injury where calcium depletion in the culture media was achieved using calcium chelators (Huser *et al.* 2007). An external mechanical stimulus increases intracellular calcium in chondrocytes through mechanosensitive ion channels and the response is abolished by removing extracellular calcium from the media (Guilak *et al.* 1999;Roberts *et al.* 2001). Altered intracellular calcium homeostasis is thought to activate a complex cascade of events involving further calcium release from the endoplasmic reticulum and mitochondria, with eventual downstream activation of

intracellular enzymes such as caspases, known to regulate chondrocyte death (Huser *et al.* 2007). It is hypothesised that depletion of external calcium inhibits these pathways of mechanotransduction that potentiate cell death and may explain the chondroprotective responses observed in articular cartilage mechanically injured in calcium-free media.

The increase in superficial zone chondrocyte death at progressively higher concentrations of calcium chloride in calcium-rich media (2-20 mM) further supports the primary hypothesis, but the findings contrast with other experiments where an increase in calcium concentration of up to 10 mM in the extracellular medium had no effect on chondrocyte death (Mansfield *et al.* 2003;Huser *et al.* 2007). These differences may be explained due to changes in medium osmolarity that occur with the addition of calcium chloride. Medium osmolarity increases by 3 mOsm for every millimole of added calcium chloride. A higher medium osmolarity significantly protects *in situ* chondrocytes from mechanical injury (Chapter 3). Hence, an increase in chondrocyte death at a higher medium calcium concentration may only be detected by controlling for the confounding effects of increased medium osmolarity. In the experiments presented here, medium osmolarity was maintained at 330 mOsm and may therefore have increased the sensitivity in detecting the increase in chondrocyte death at higher medium calcium concentrations.

These data do not support the secondary hypothesis that culture of injured articular cartilage in calcium-rich media increases *in situ* chondrocyte death. Instead, there was an increase in cell death over 7 days in calcium-free media. With two-plane

CLSM the spatial distribution of this increase in chondrocyte death was clearly defined – cell death progressed from the articular surface downwards to the middle and deep zones within injured cartilage (**Figure 4.1**). Further, there was no progression of cell death at the articular surface and no increase in cell death within the uninjured region of the articular surface, irrespective of medium calcium concentration (**Figure 4.2**). The findings compare with those reported in equine cartilage subjected to impact trauma since there was no significant increase in chondrocyte death in the superficial zone (as quantified in coronal histological sections using a TUNEL assay) with medium calcium concentration increased up to 10 mM (Huser *et al.* 2007). However, the study also reported a decrease in chondrocyte death associated with a reduction in extracellular calcium, in contrast to the findings of increased cell death in calcium-free media noted in the current experiments. This difference may be explained by the shorter duration (48 hours) of explant culture in the published study (Huser *et al.* 2007). An increase in cell death in the deeper zones of cartilage noted in the experiments presented in this thesis only occurred in explants cultured in calcium-free media over 7 days, suggesting that extracellular calcium may be important for maintaining calcium homeostasis and cell viability over a longer period of time in injured cartilage. This delayed increase in chondrocyte death in calcium-free media also supports the use of calcium supplementation in media used during cartilage explant culture.

In conclusion, exposure of articular cartilage to calcium-free media significantly decreased superficial zone chondrocyte death following mechanical injury compared with exposure to calcium-rich media (2-20 mM). In calcium-rich media, the extent of

chondrocyte death increased with increasing medium calcium concentration but remained localised to the superficial zone of articular cartilage over 7 days. However, in calcium-free media, there was an increase in chondrocyte death within deeper zones of injured articular cartilage over 7 days.

# CHAPTER 5

# CHONDROCYTE DEATH IN INJURED BOVINE ARTICULAR CARTILAGE - EFFECT OF INCREASING THE OSMOLARITY OF 0.9% SALINE AND HARTMANN'S SOLUTIONS

## 5.1 HYPOTHESES

### Primary Hypothesis

Exposure of articular cartilage to high osmolarity 0.9% Saline and Hartmann's solutions decreases *in situ* chondrocyte death in the superficial zone following mechanical injury (within hours).

### Secondary Hypothesis

Exposure of articular cartilage to Hartmann's solution increases *in situ* chondrocyte death in the superficial zone compared with 0.9% Saline following mechanical injury (within hours).

## 5.2 CHAPTER SUMMARY

The objective was to determine whether the chondroprotective effects of high culture medium osmolarity in injured bovine articular cartilage can be reproduced using irrigation solutions commonly used during open and arthroscopic articular surgery, such as 0.9% Saline and Hartmann's solution. Using CLSM, *in situ* chondrocyte death at the injured cartilage edge was spatially quantified as a function of the osmolarity of 0.9% Saline and Hartmann's solutions at 2.5 hours. Increasing the osmolarity of 0.9% Saline and Hartmann's solutions to 600 mOsm, significantly decreased *in situ* chondrocyte death in the superficial zone of injured cartilage ($p<0.05$). Compared with 0.9% Saline, Hartmann's solution was associated with greater chondrocyte death in the superficial zone of injured cartilage ($p=0.03$), but the difference was not significant if the osmolarity of both solutions was increased to 600 mOsm. In conclusion, increasing the osmolarity of 0.9% Saline and Hartmann's

solutions to 600 mOsm was chondroprotective in a surgically relevant bovine model of cartilage injury.

## 5.3 CHAPTER INTRODUCTION

During open and arthroscopic articular surgery, synovial fluid, which normally maintains the physiological environment within a joint, is replaced by a joint irrigation solution for the duration of the procedure. Several studies have investigated the effects of different joint irrigation solutions on articular cartilage in order to identify a solution that optimises cartilage biosynthetic function. In an *ex vivo* experiment bovine articular cartilage slices were incubated with $^{35}SO_4$ in various commercially available solutions and assayed for incorporated radioactivity indicating proteoglycan synthesis in cartilage (Reagan *et al.* 1983). In the study, 0.9% Saline suppressed proteoglycan synthesis after two hours while Hartmann's solution maintained metabolic activity within cartilage for up to eight hours. In contrast, in a similar study using anatomically intact rat patellae to avoid the problem of proteoglycan loss from the surface of detached articular cartilage slices, 0.9% Saline and Hartmann's solution suppressed proteoglycan synthesis to the same extent after 16 hours (Bulstra *et al.* 1994). Histological and electron microscopic evaluation of articular cartilage after exposure to these solutions did not find any alterations in cartilage ultrastructure after *in vivo* exposure in animal models for up to five days (Yang, Cheng, & Shen 1993;Gunal *et al.* 2000). While these studies have investigated the metabolic and structural effects of various irrigation solutions on uninjured articular cartilage, the effect on chondrocyte viability in mechanically injured articular cartilage has not been reported.

In the experiments detailed in Chapters 3 and 4, *in situ* chondrocyte viability in the superficial zone of injured bovine cartilage was influenced by medium osmolarity and calcium concentration. Exposure of articular cartilage to media with a low osmolarity increased the extent of *in situ* superficial zone chondrocyte death, while exposure to a high osmolarity was chondroprotective (Chapter 3). These chondroprotective effects were evident within hours and in addition, there was no increase in cell death from 2.5 hours to 7 days suggesting (1) exposure of articular cartilage to a high medium osmolarity did not compromise *in situ* chondrocyte function in the long term and (2) investigation into such chondroprotection should focus on the early (within hours) effects of high medium osmolarity. Further, exposure of articular cartilage to calcium-free media (~0 mM) also significantly reduced superficial zone chondrocyte death within hours after mechanical injury compared with exposure to calcium-rich media (Chapter 4).

The experiments in Chapters 3 and 4 were performed by modulating the osmolarity and calcium concentration of standard culture medium (DMEM). The chondroprotective effects demonstrated in the bovine model of cartilage injury have relevance to the composition of joint irrigation solutions used during articular surgery, such as Saline (0.9%) and Hartmann's solution (Reagan *et al.* 1983). The mean osmolarity of these solutions ranges between 250-300 mOsm and is much lower than that of human synovial fluid which has an osmolarity of approximately 400 mOsm (Baumgarten *et al.* 1985). The calcium concentrations of these solutions also differ. While Saline (0.9%) contains negligible concentration of calcium ions, Hartmann's solution contains 2mM of calcium. The presence of calcium in

Hartmann's solution is thought to support chondrocyte metabolism better than solutions without added calcium - such as 0.9% Saline - which have previously been considered to be non-physiological (Reagan *et al.* 1983). *In situ* chondrocytes must therefore experience a transient change in their extracellular environment during the surgical procedure depending on the composition of the irrigation solution used. Based on the findings from experiments detailed in Chapters 3 and 4, optimising the composition of the irrigation solution may therefore provide a potential strategy for decreasing the extent of chondrocyte death from mechanical injury during articular surgery.

The objective was to determine whether the chondroprotective effects of high culture medium osmolarity in injured bovine articular cartilage can be reproduced using irrigation solutions such as 0.9% Saline and Hartmann's solution. The primary hypothesis was that exposure of articular cartilage to high osmolarity 0.9% Saline and Hartmann's solutions decreases *in situ* chondrocyte death in the superficial zone following mechanical injury (within hours). The secondary hypothesis was that exposure of articular cartilage to Hartmann's solution (which contains a high concentration of calcium ions) increases *in situ* superficial zone chondrocyte death compared with 0.9% Saline following mechanical injury (within hours). Using CLSM and the established bovine model of cartilage injury, *in situ* chondrocyte death at the injured cartilage edge has been spatially defined and quantified as a function of the osmolarity of 0.9% Saline and Hartmann's solutions at 2.5 hours.

## 5.4 RESULTS

### 5.4.1 Spatial distribution of cell death

Coronal CLSM reconstructions of the full thickness of injured cartilage demonstrated chondrocyte death localised mainly within the superficial zone, with relative sparing of the middle and deeper zones (**Figures 5.1 and 5.3**). These findings were similar to those detailed for the coronal CLSM reconstructions of injured cartilage in Chapters 3 and 4.

Axial CLSM reconstructions imaging the superficial zone 'top-down' from the articular surface indicated a 'band' of cell death at the scalpel injured edge for the range of medium osmolarities (100-600 mOsm). The extent of the band of cell death was greatest for explants exposed to low osmolarity solutions and least for explants exposed to modified, high osmolarity solutions (**Figures 5.2 and 5.4**). *In situ* chondrocytes within the adjacent uninjured region of the articular surface were unaffected by exposure to 0.9% Saline or Hartmann's solution for the range of medium osmolarities (0-600 mOsm), with no visible cell death in this region. These findings were again similar to those detailed for the axial CLSM reconstructions of injured cartilage in Chapters 3 and 4.

### 5.4.2 Percentage cell death

#### 5.4.2.1 With 0.9% Saline osmolarity varied from 100-600 mOsm

Percentage cell death quantified from coronal CLSM reconstructions ($PCD_{FT}$) - imaging the full thickness of articular cartilage - indicated that cell death mainly occurred in the superficial zone (~first 100 μm from the articular surface) of injured

cartilage for explants exposed to 100, 285 (control) and 500 mOsm saline solutions, with relative sparing of the middle and deep zones (N=3, n=9**, Figure 5.1**). For explants exposed to the 600 mOsm saline, $PCD_{FT}$ within the first 100 μm of the articular surface was similar to the $PCD_{FT}$ at depths >100 μm (N=3, n=3, **Figure 5.1**).

Since coronal CLSM reconstructions indicated that alterations in saline osmolarity affected *in situ* chondrocyte death mainly in the upper 100μm of articular cartilage, percentage cell death was compared in axial CLSM reconstructions exclusively imaging the band of *in situ* chondrocyte death in the superficial zone of articular cartilage. Compared to the control explants exposed to 0.9% Saline (285 mOsm), the percentage cell death was greatest for the low osmolarity (100 mOsm) saline solution and least for the high osmolarity (600 mOsm) saline solution (N=3, n=36, observations from three replicates averaged for each data point, one-way ANOVA for increasing saline osmolarity, p=0.04, **Figure 5.2**).

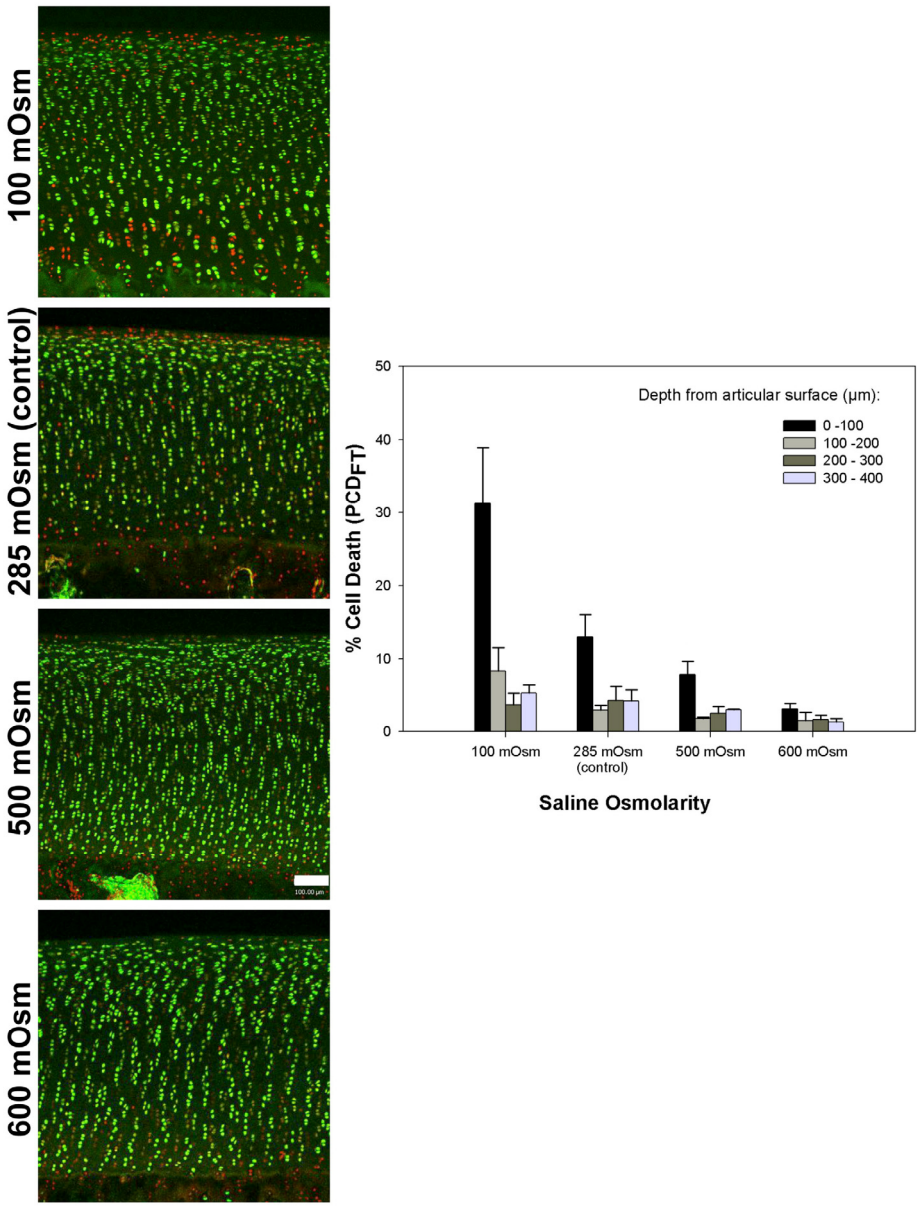


**Figure 5.1: Panels show coronal CLSM reconstructions of the injured articular cartilage. Bar chart shows the corresponding pooled data for $PCD_{FT}$ as a function of increasing saline osmolarity (100-600 mOsm)**

*In situ* chondrocyte death was mainly localised near the articular surface (i.e. superficial zone, 0-100 µm depth-interval) for explants exposed to 100, 285 (control) and 500 mOsm saline solutions, with relative sparing of the middle and deep zones. At 600 mOsm, there was chondroprotection in this region of injured articular cartilage (white bar=100µm).

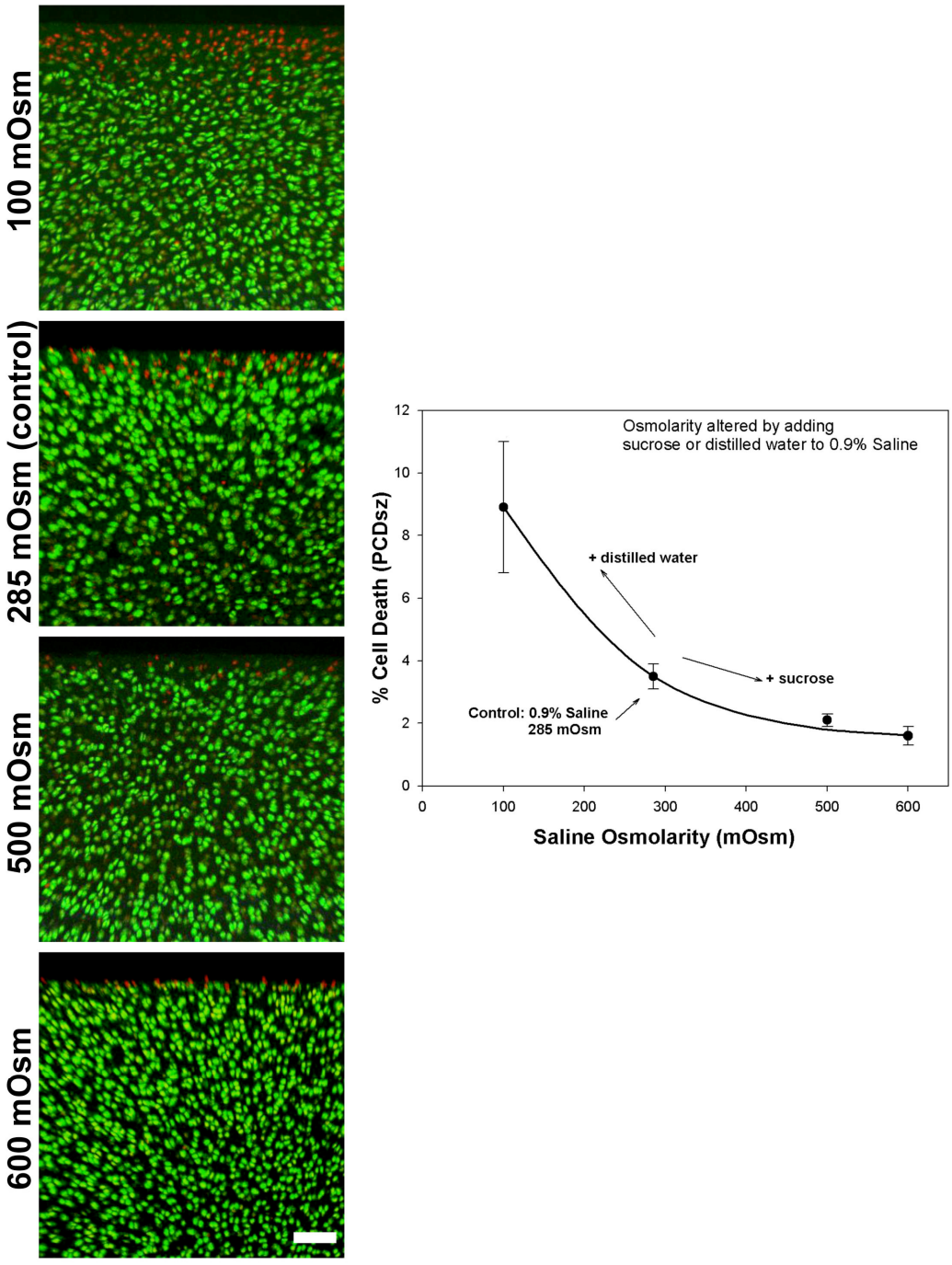


**Figure 5.2: Panels show axial CLSM reconstructions of the injured cartilage edge. Graph shows the corresponding pooled data for $PCD_{SZ}$ as a function of increasing saline osmolarity (100-600 mOsm)**

The 'band' of superficial zone chondrocyte death at the cut cartilage edge (PI stained red nuclei) decreased significantly for explants exposed to an increasing osmolarity of the saline solution (ANOVA for increasing saline osmolarity, p=0.04, white bar = 100 μm).

#### 5.4.2.2 Comparing 0.9% Saline, Hartmann's and modified high osmolarity solutions

In separate experiments, injured explants were exposed to the proprietary (control) solutions of Hartmann's (255 mOsm), 0.9% Saline (285 mOsm), and modified, high osmolarity (600 mOsm) preparations of Hartmann's and 0.9% Saline. $PCD_{FT}$ once again indicated that cell death mainly occurred in the superficial zone (~ first 100 μm from the articular surface) of injured cartilage for explants exposed to the control solutions of Hartmann's (255 mOsm) and 0.9% Saline (285 mOsm), with relative sparing of the middle and deep zones (N=3, n=6, **Figure 5.3**). For explants exposed to the modified, high osmolarity (600 mOsm) solutions of Hartmann's and 0.9% Saline, $PCD_{FT}$ within the first 100 μm of the articular surface was similar to $PCD_{FT}$ at depths >100 μm (N=3, n=6, **Figure 5.3**).

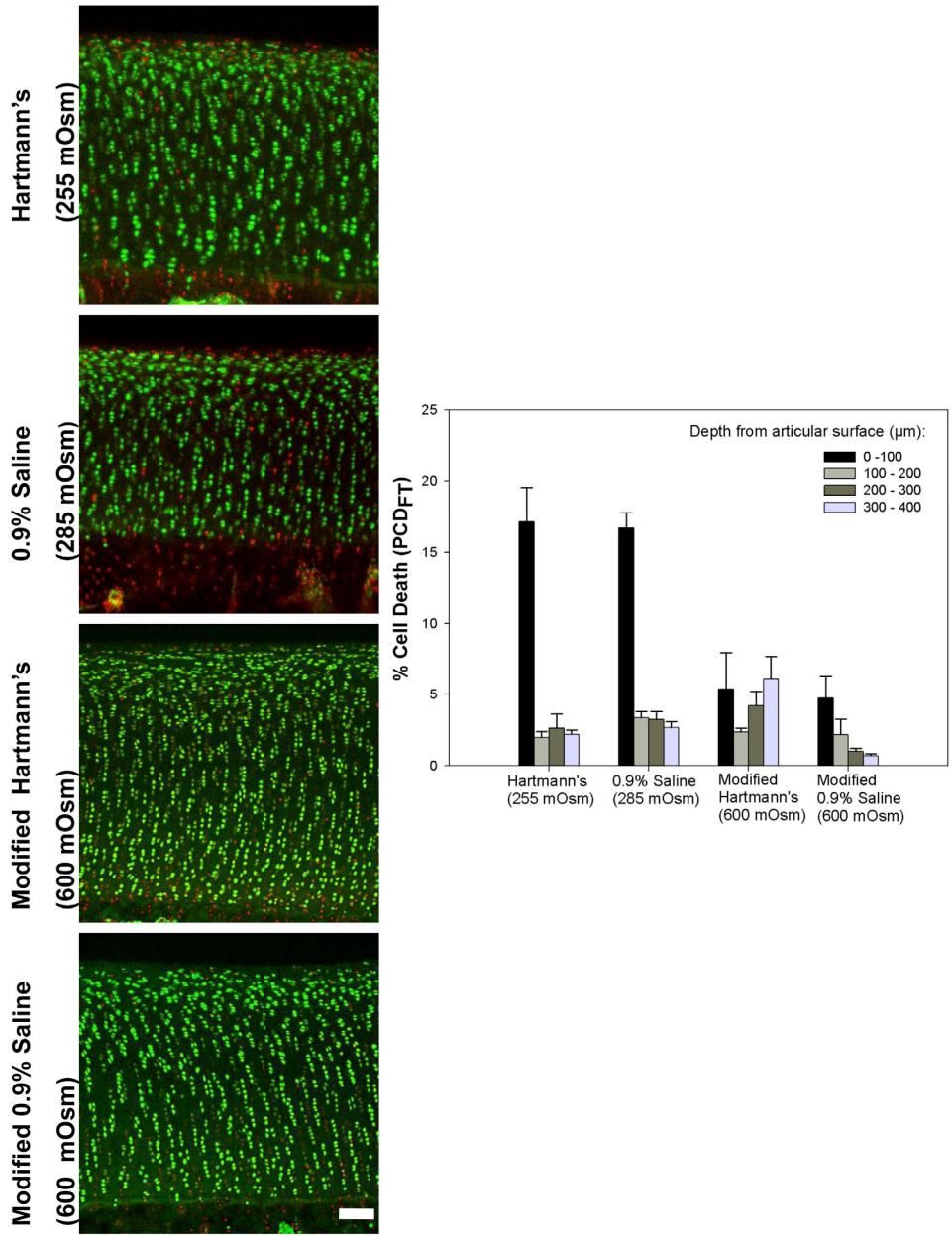


**Figure 5.3: Panels show coronal CLSM reconstructions of the injured cartilage edge. Bar chart shows corresponding pooled data for $PCD_{FT}$ comparing the extent of cell death between 0.9% Saline and Hartmann's solutions**

*In situ* chondrocyte death was mainly localised near the articular surface (i.e. superficial zone, 0-100 µm depth-interval) for explants exposed to control solutions of Hartmann's (255 mOsm) and 0.9% Saline (285 mOsm), with relative sparing of the middle and deep zones. For explants exposed to the modified, high osmolarity (600 mOsm) solutions of Hartmann's and 0.9% Saline, there was chondroprotection in this region of injured articular cartilage (white bar = 100 µm).

Since coronal CLSM reconstructions indicated that alterations in medium osmolarity affected *in situ* chondrocyte death mainly in the upper 100μm of articular cartilage, percentage cell death was compared in axial CLSM reconstructions exclusively imaging the band of *in situ* chondrocyte death in the superficial zone of articular cartilage. $PCD_{SZ}$ was significantly greater for explants exposed to the control solution (255 mOsm) of Hartmann's compared to the control solution (285 mOsm) of 0.9% Saline (N=3, n=18, observations from three replicates averaged for each data point, paired t-test, p=0.03, **Figure 5.4**). Compared to their respective control explants, $PCD_{SZ}$ significantly decreased for explants exposed to the modified, high osmolarity (600 mOsm) solutions of Hartmann's and 0.9% Saline (N=3, n=18, observations from three replicates averaged for each data point, p=0.006 for 600 mOsm vs. 255 mOsm Hartmann's solutions; N=3, n=18, observations from three replicates averaged for each data point, p=0.01 for 600 mOsm vs. 285 mOsm 0.9% Saline solutions, paired t-tests). Further, there was no significant difference in $PCD_{SZ}$ between explants exposed to the modified, high osmolarity (600 mOsm) solutions of Hartmann's and 0.9% Saline (N=3, n=18, observations from three replicates averaged for each data point, p=0.5, paired t-test).

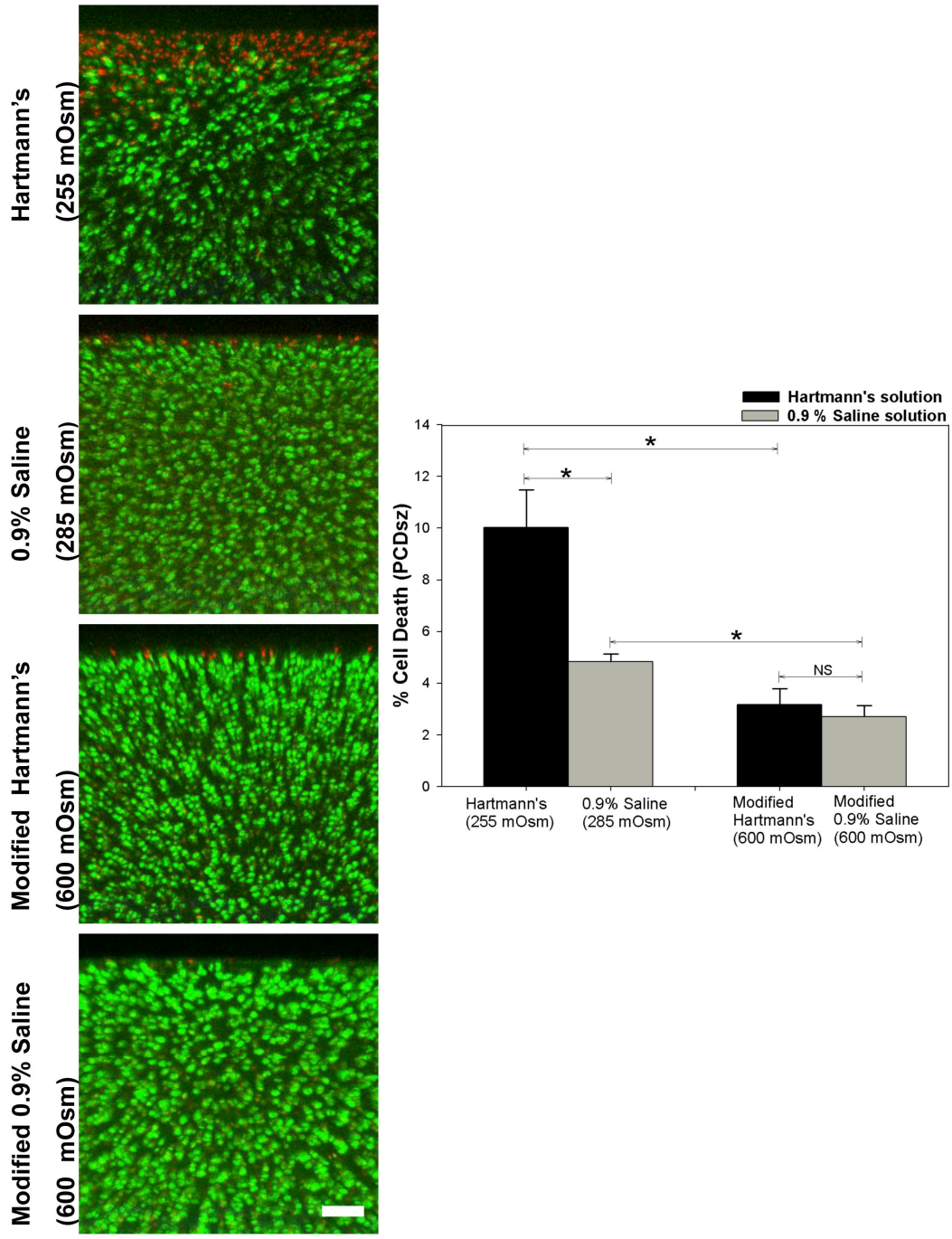


**Figure 5.4: Panels show axial CLSM reconstructions of the injured cartilage edge. Bar chart shows corresponding pooled data for $PCD_{SZ}$ comparing the extent of cell death between 0.9% Saline, Hartmann's and modified high osmolarity (600 mOsm) solutions**

The 'band' of superficial zone chondrocyte death at the cut cartilage edge decreased after increasing the osmolarity of Hartmann's (255 mOsm) and 0.9% Saline (285 mOsm) solutions to 600 mOsm (*=$p<0.05$ for paired comparisons, NS = Not significant ($p>0.05$), white bar = 100 μm).

## 5.5 CHAPTER DISCUSSION

Two important findings can be surmised from the study. Firstly, increasing the osmolarity of 0.9% Saline (285 mOsm) and Hartmann's (255 mOsm) solutions to 600 mOsm significantly decreased *in situ* chondrocyte death in the superficial zone following a mechanical (scalpel) injury to bovine articular cartilage. Secondly, Hartmann's solution (255 mOsm) was associated with greater chondrocyte death in the superficial zone of injured cartilage compared with 0.9% Saline (285 mOsm), but the difference was not significant if the osmolarity of both solutions was increased to 600 mOsm. These *in vitro* experiments also confirm that the chondroprotective effects of increasing the osmolarity of standard culture media (DMEM) can be reproduced using joint irrigation solutions in common use during articular surgery.

Findings from this study support our primary hypothesis that increasing the osmolarity of 0.9% Saline and Hartmann's solutions decreases *in situ* chondrocyte death in the superficial zone following mechanical injury. These data suggest that cells in the superficial zone are not only most susceptible to mechanical injury, but also most sensitive to the chondroprotective effects exerted by high osmolarity solutions. Sucrose was used for increasing the osmolarity of the solutions as the disaccharide is impermeable across animal cell membranes and is not metabolised by articular chondrocytes (Fell 1969;Fell *et al.* 1969). An alternative would be to use sodium chloride to increase the osmolarity of the solutions as it has similar, but not identical effects on matrix synthesis rates compared to sucrose when used at identical osmolarities (Urban *et al.* 1993). The small difference is probably due to differential effects of sucrose and sodium on intracellular composition (Urban *et al.* 1993).

Sucrose addition would decrease chondrocyte volume thereby raising intracellular ion concentrations. When sodium chloride is used as an osmotic replacement, it is likely that in addition to a decrease in chondrocyte volume, there will be a further increase in the intracellular sodium ion concentration potentially leading to altered activity of sodium-dependent membrane transporters, particularly those involved in intracellular pH regulation (Wilkins & Hall 1992). Thus, interpretation of the results using sucrose is easier as only chondrocyte volume is altered, without secondary changes in cell sodium levels.

In this experiment, axial CLSM reconstructions of the articular surface indicated that Hartmann's solution (255 mOsm) was associated with greater superficial zone chondrocyte death at the cut edge compared with 0.9% Saline (285 mOsm, **Figure 5.4**), lending support to the secondary hypothesis. The difference in the extent of cell death between these two solutions was not completely accounted for by the lower osmolarity of the Hartmann's solution. In addition to sodium chloride, Hartmann's solution also contains 2 mM of calcium as a chloride salt. The presence of calcium in Hartmann's solution is believed to support chondrocyte metabolism better than solutions without added calcium such as 0.9% saline (Reagan *et al.* 1983). However, as detailed in the experiments performed in Chapter 4, a reduction in calcium in the extracellular medium also decreased chondrocyte death after mechanical injury, possibly through the prevention of an increase in cytoplasmic calcium (Huser *et al.* 2007). It is hypothesised that the greater chondrocyte death associated with Hartmann's solution compared to 0.9% Saline is not only due to the lower osmolarity of the Hartmann's solution, but also due to the calcium present in its preparations.

Interestingly, the difference in the extent of cell death between Hartmann's solution (255 mOsm) and 0.9% Saline (285 mOsm) was not significant if the osmolarity of both solutions was increased to 600 mOsm, suggesting that a higher osmolarity not only elicited a relatively greater chondroprotective effect for the Hartmann's solution, but also negated the additional cell death that may be attributed to the elevated calcium.

In conclusion, increasing the osmolarity of 0.9% Saline (285 mOsm) and Hartmann's solution (255 mOsm) to 600 mOsm was chondroprotective in a surgically relevant bovine model of mechanical cartilage injury.

# CHAPTER 6

# CHONDROCYTE DEATH IN INJURED HUMAN CARTILAGE - CHONDROPROTECTION WITH A MODIFIED 0.9% SALINE SOLUTION

## 6.1 HYPOTHESIS

Exposure of human articular cartilage to hyperosmotic saline (0.9%, 600 mOsm) decreases *in situ* chondrocyte death following mechanical injury compared with exposure to normal saline (0.9%, 285 mOsm).

## 6.2 CHAPTER SUMMARY

The rationale for this experiment was based on the chondroprotective effects of high medium osmolarity established in the bovine model of cartilage injury (Chapters 3 and 5). The objective was to determine if exposure of human articular cartilage to hyperosmotic saline (0.9%, 600 mOsm) decreased *in situ* chondrocyte death following a standardised mechanical injury (scalpel cut) compared with exposure to normal saline (0.9%, 285 mOsm). Non-degenerate human cartilage explants were exposed to normal (control) and hyperosmotic 0.9% saline solutions for 5 minutes prior to the mechanical injury (to allow *in situ* chondrocytes to respond to the altered osmotic environment). The injured explants were incubated for a further 2.5 hours in the same solutions following the mechanical injury. Using CLSM, *in situ* chondrocyte death at the injured cartilage edge has been spatially defined and quantified in human articular cartilage as a function of the 0.9% saline osmolarity at 2.5 hours. There was a six-fold ($p<0.05$) decrease in chondrocyte death following mechanical injury in the superficial zone of human articular cartilage exposed to hyperosmotic saline (0.9%, 600 mOsm) compared to normal saline (0.9%, 285 mOsm). These data suggest that increasing the osmolarity of joint irrigation solutions used during open and arthroscopic articular surgery may decrease chondrocyte death from surgical injury and could promote integrative cartilage repair in human tissue.

## 6.3 CHAPTER INTRODUCTION

In the bovine model of cartilage injury, a decrease in medium osmolarity increased chondrocyte death following mechanical injury, while an increase in medium osmolarity was chondroprotective. Further, increasing the osmolarity of 0.9% Saline from 285 mOsm to 600 mOsm resulted in the greatest decrease in chondrocyte death following mechanical injury. Although the precise mechanism for the chondroprotection remains to be established, it is likely that chondrocytes swell at low extracellular osmolarity and become more susceptible to mechanical injury (Bush *et al.* 2001b;Bush *et al.* 2005b). Conversely, chondrocytes shrink at high extracellular osmolarity and are protected from mechanical injury (Bush *et al.* 2001b;Bush *et al.* 2005b). These cellular responses (volume changes and death) are most marked in the superficial zone of articular cartilage – the layer of articular cartilage essential for maintaining a smooth, low friction, lubricating surface (Buckwalter *et al.* 1998b;Hunziker 2002) and representing a region of greatest cell density (Hunziker *et al.* 2002).

As previously discussed (Chapter 5), during articular surgery the synovial fluid - which normally maintains the physiological milieu within a joint - is drained and replaced by an irrigation solution such as 0.9% Saline, with an osmolarity ranging between 250-300 mOsm. However, normal human synovial fluid osmolarity is approximately 400 mOsm (Baumgarten *et al.* 1985). *In situ* human articular chondrocytes therefore experience a marked decrease in extracellular osmolarity during the surgical procedure and are then more likely to be susceptible to a subsequent mechanical injury. During open and arthroscopic articular surgery,

cartilage is subjected to such mechanical insults frequently from the surgical instruments and implants. The chondroprotection afforded by high medium osmolarity established in the bovine cartilage system merits evaluation in human articular cartilage, as the use of high osmolarity joint irrigation solutions during reconstructive articular surgery may decrease chondrocyte death from mechanical injury, increase the viable cell population within the injured cartilage edge and hence, promote integrative cartilage repair (Gilbert *et al.* 2009).

The aim was to determine whether the chondroprotective effects of the hyperosmotic saline solution (0.9%, 600 mOsm) in injured bovine cartilage could be reproduced in human articular cartilage. The hypothesis was that exposure of human articular cartilage to hyperosmotic saline (0.9%, 600 mOsm) decreases *in situ* chondrocyte death following a full thickness mechanical injury (using a scalpel) compared with exposure to normal saline (0.9%, 285 mOsm). Using CLSM, *in situ* chondrocyte death at the injured cartilage edge has been spatially defined and quantified in human articular cartilage as a function of the 0.9% saline osmolarity at 2.5 hours.

## 6.4 RESULTS

### 6.4.1 Spatial distribution of cell death

Axial CLSM reconstructions visualised *in situ* superficial zone chondrocytes 'top-down' from the articular surface at the scalpel cut cartilage edge. The spatial distribution of cell death within axial CLSM reconstructions was similar to bovine cartilage with a 'band' of cell death at the scalpel injured edge, with sparing of the articular chondrocytes within the adjacent uninjured region of the articular surface

(**Figure 6.1**). The extent of the band of cell death was greater for explants exposed to normal saline (0.9%, 285 mOsm) than those exposed to hyperosmotic saline (0.9%, 600 mOsm).

Since, human articular cartilage exhibits zonal heterogeneity (Hunziker *et al.* 2002), the cellular responses to mechanical injury were also imaged within its full thickness. These coronal CLSM reconstructions visualised *in situ* chondrocytes within different zones at the scalpel cut cartilage edge and demonstrated the heterogeneous spatial distribution of chondrocytes - cells in the superficial zone were orientated parallel to the articular surface, while cells in the middle zone and deep zones were orientated more vertically in columns (**Figure 6.2**). Human articular cartilage obtained from the knee joint was much thicker (2-4 mm) than bovine cartilage from the metacarpophalangeal joint (< 1mm). Although this corresponded to a greater thickness of the superficial, middle and deep zones in human cartilage, the zonal organisation was similar to bovine cartilage.

### 6.4.2 Percentage cell death

Axial CLSM reconstructions showed a significant decrease in percentage cell death at the injured cartilage edge (representing superficial zone chondrocytes at the articular surface) for explants exposed to hyperosmotic saline (0.9%, 600 mOsm) compared with the control solution of normal saline (0.9%, 285 mOsm, N=4, n=8, p=0.01, paired t-test, **Figure 6.1**).

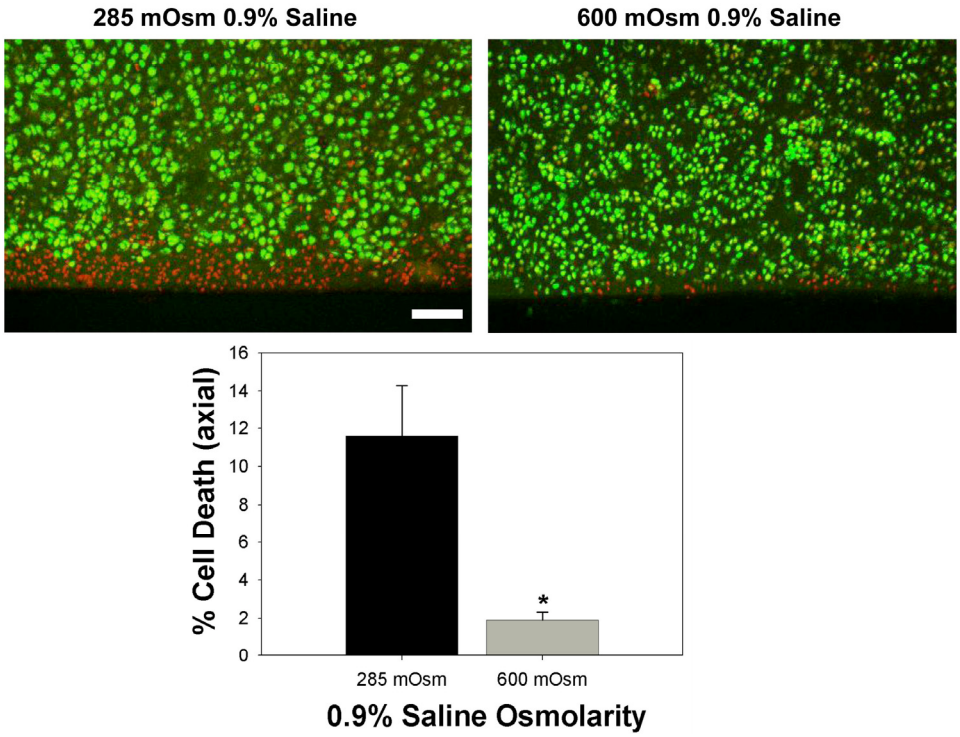


**Figure 6.1: Axial CLSM reconstructions show the decrease in superficial zone cell death at the articular surface for explants exposed to 600 mOsm 0.9% saline**

Note the 'band' of cell death at the injured cartilage edge in both images. Bar chart shows pooled data from four different patients with a significant decrease in percentage cell death for explants exposed to 600 mOsm saline compared with 285 mOsm saline (dead cells stain red with PI, live cells stain green with CMFDA, x10 power CLSM, *$p<0.05$ versus 285 mOsm, white bar =100 μm).

Coronal CLSM reconstructions showed a significant decrease in percentage cell death in the superficial zone of injured cartilage for explants exposed to hyperosmotic saline (0.9%, 600 mOsm) compared with the control solution of normal saline (0.9%, 285 mOsm, N=4, n=8, p=0.04, paired t-test, **Figure 6.2**). The differences in percentage cell death in the middle and deep zones were not significant for explants exposed to the two different solutions (N=4, n=8, p=0.5, paired t-tests for middle and deep zones, **Figure 6.2**).

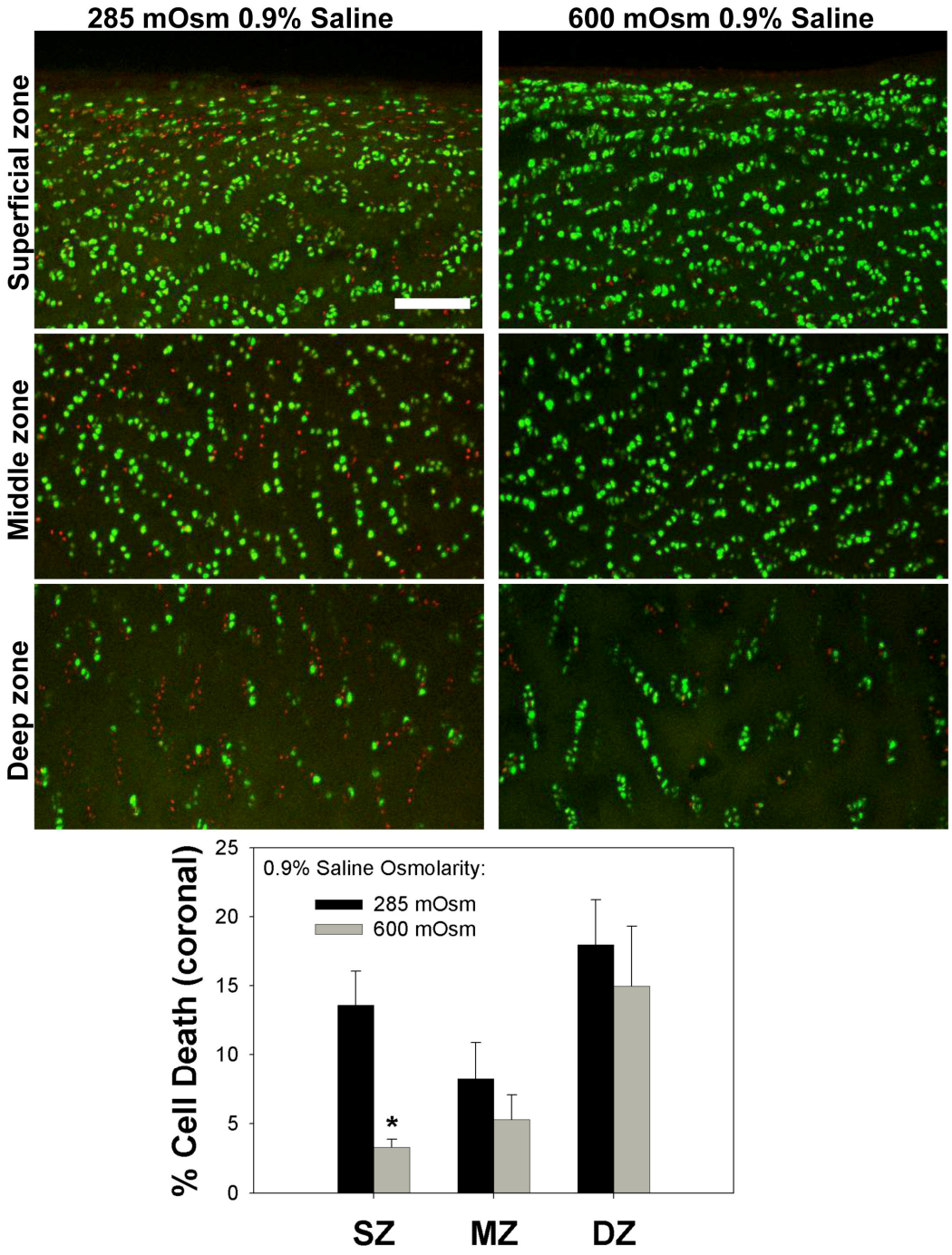


**Figure 6.2: Coronal CLSM reconstructions of the injured cartilage edge show the decrease in superficial zone cell death for explants exposed to 600 mOsm 0.9% saline**

Note the heterogeneous zonal distribution of chondrocytes. The bar chart shows pooled data from four different patients with a significant decrease in percentage cell death in the superficial zone for explants exposed to 600 mOsm saline compared with 285 mOsm saline (x10 power CLSM, *$p<0.05$ versus 285 mOsm, white bar =100 μm).

### 6.4.3 Cartilage cell density

The cell density (total number of chondrocytes/$mm^3$) in human articular cartilage explants is shown in **Table 6.1**. There was no significant difference in cell density (number of chondrocytes/$mm^3$) between explants exposed to the 285 mOsm or 600 mOsm 0.9% saline solutions (N=4, p=0.9 for cell density within the full thickness of articular cartilage, p= 0.7 for cell density with the superficial zone of articular cartilage, unpaired t-tests). Superficial zone cell density was significantly greater than the mean cell density within the full thickness of articular cartilage for explants in each experimental group (N=4, p=0.01 for normal saline, p<0.001 for hyperosmotic saline, unpaired t-tests)**.**

| | **Cell density in articular cartilage (No. of cells/$mm^3$)** | |
|---|---|---|
| | **285 mOsm 0.9% saline** | **600 mOsm 0.9% saline** |
| **Full thickness** | 9105 ± 1346 | 9382 ± 1192 |
| **Superficial zone** | 27579 ± 3319 | 28900 ± 351 |

**Table 6.1: Comparison of cell density within articular cartilage for explants exposed to 285 mOsm and 600 mOsm 0.9% Saline**

No significant difference in cell density (number of chondrocytes/$mm^3$ ± standard error) between explants exposed to the 285 mOsm or 600 mOsm 0.9% saline solutions (p=0.9 for full thickness, p= 0.7 for superficial zone, unpaired t-tests). Note also the superficial zone cell density is significantly greater than the mean cell density within the full thickness of articular cartilage (p=0.01 for 285 mOsm saline, p<0.001 for 600 mOsm saline, unpaired t-tests).

## 6.5 CHAPTER DISCUSSION

The findings from these experiments show that *in situ* chondrocyte death following a mechanical (scalpel) injury was significantly decreased (approximately six-fold) in the superficial zone of human articular cartilage exposed to hyperosmotic saline (0.9%, 600 mOsm) compared with exposure to normal saline (0.9%, 285 mOsm). The similarities in the *ex vivo* bovine and human models of mechanical injury in relation to the spatial distribution of live and dead *in situ* chondrocytes within cartilage and the preferential chondroprotection of superficial zone articular chondrocytes, supports the view that chondroprotective parameters tested in a bovine cartilage system may be extrapolated to human cartilage. This investigation in human articular cartilage therefore extends findings from the bovine model and provides evidence for increasing the osmolarity of joint irrigation solutions used during open and arthroscopic articular surgery to promote chondrocyte survival following surgical injury.

As previously discussed (Chapter 3, section 3.5), it is hypothesised that the chondroprotective mechanisms of high osmolarity involve a decrease in chondrocyte volume due to water efflux from cells that protects from the mechanical insult (Bush *et al.* 2005b). Disaccharides such as sucrose may prove ideal for increasing the osmolarity of joint irrigation solutions as they are not toxic to soft tissues and maintain an extracellular osmotic pressure gradient without being metabolised by articular chondrocytes (Fell 1969;Fell *et al.* 1969).

The majority of research concerning the responses of articular chondrocytes to mechanical injury is based on animal models which have utilised non-degenerate articular cartilage. Findings from animal models need to be corroborated in human articular cartilage to facilitate extrapolation to the clinical scenario. However, the supply of non-degenerate human articular cartilage for such experimental work is very limited. Although osteochondral tissue discarded at total knee replacement is readily available, the amount of microscopically and macroscopically non-degenerate human articular cartilage (International Cartilage Repair Society (ICRS) Grade 0 (Mainil-Varlet *et al.* 2003;Huntley *et al.* 2005c)) harvested from the resected specimens is small. With limited availability of such human tissue, the techniques of experimental cartilage injury, cell viability staining, CLSM imaging, automated quantification as well as the osmotic parameters for chondroprotection defined in the bovine model of cartilage injury facilitated testing of the key hypotheses in human cartilage.

Even if human articular cartilage appeared macroscopically normal, the tissue was graded according to the ICRS system using CLSM (Huntley *et al.* 2005c) to ensure the integrity of the superficial zone microscopically and distinguish ICRS Grade 0 (normal cartilage) from ICRS Grade 1 articular cartilage (which exhibits a loss of integrity at the articular surface with a variable associated loss in superficial zone chondrocytes). This was important because the initial bovine model of cartilage injury indicated that *in situ* chondrocytes in the superficial zone were most sensitive to the chondroprotective effects of a high medium osmolarity - the integrity of the

entire superficial zone in human cartilage (present only in ICRS Grade 0 cartilage) was therefore crucial in evaluating any protection from mechanical injury.

For the purposes of this experiment, non-degenerate human articular cartilage (ICRS grade 0) was harvested from an otherwise degenerate joint. It was therefore important to consider whether non-degenerate articular chondrocytes within an otherwise degenerate joint exhibit osmotic as well as chondroprotective responses that are similar to articular chondrocytes from a healthy joint. The biophysical parameters and the responses to mechanical injury of non-degenerate human articular cartilage harvested from an otherwise degenerate joint have been demonstrated to be similar to normal articular cartilage, provided the tissue is macroscopically and microscopically ICRS Grade 0 (Huntley *et al.* 2005c). Moreover, in human articular cartilage, the passive osmotic properties of non-degenerate and degenerate human articular chondrocytes in response to changes in extracellular osmolarity are in fact similar, although the magnitude of cell volume change is greater in some degenerate articular chondrocytes due to the reduced interstitial osmolarity occurring with cartilage degeneration (Bush *et al.* 2005a). It would therefore be reasonable to suggest that in this experiment (where articular cartilage was confirmed to be macroscopically and microscopically ICRS Grade 0), the osmotic sensitivity of *in situ* articular chondrocytes would be similar to normal articular cartilage and findings from this investigation may be extrapolated to the clinical scenario where reconstructive surgery is performed mainly on non-degenerate cartilage.

Low power (x10) CLSM, live/dead fluorescent probes and validated intensity thresholding techniques for automated image analysis (Jomha *et al.* 2003;Lin *et al.* 2005) were used to quantify *in situ* chondrocyte death in three-dimensions at the injured cartilage edge. The uptake of the fluorescent indicators (CMFDA (10μM) and PI (10μM)) by *in situ* human articular chondrocytes was comparable to bovine tissue and provided adequate evaluation of cellular viability with CLSM. The penetration of the fluorescent dyes into human tissue was greater, allowing CLSM imaging to a depth of ~100μm in both axial and coronal planes (compared to a depth of ~60-80μm in bovine cartilage). In a heterogeneous, anisotropic tissue such as articular cartilage, this experiment highlights the advantages of CLSM over traditional microscopy for studying *in situ* cellular responses to mechanical injury, because it permits three-dimensional visualisation of the same tissue volume from different perspectives. For instance, human articular cartilage utilised in this experiment was much thicker than the bovine cartilage used in the animal model. Coronal CLSM images obtained at defined distances from the articular surface and the osteochondral junction controlled for the variation in cartilage thickness between explants and allowed differential assessment of *in situ* chondrocyte responses to mechanical injury within the superficial, middle and deep zones. Further, axial CLSM imaging of the same region defined the extent of superficial zone cell death at the articular surface extending proximally from the injured cartilage edge.

The use of automated cell counting techniques allowed rapid and unbiased quantification within comparatively large regions of interest which contained around 1000 individual cells. This reduced the variability associated with manual cell counts

that typically utilise smaller regions of interest with fewer cells and allowed meaningful quantification despite a relatively small study sample. Measured values of cell density were similar between explants and comparable to those reported in normal adult human articular cartilage using histological techniques (Hunziker *et al.* 2002).

The findings from these experiments in human cartilage represent the culmination of the chondroprotective strategies initially developed in the bovine model (Chapters 3, 4 and 5). Extrapolation from this human *in vitro* system to the clinical scenario must be made with caution as these findings have certain limitations. Firstly, the effects of high saline osmolarity on soft tissues such as the synovium, menisci and ligaments have not been evaluated. However, intra-articular contrast media used in investigative radiology typically have a higher osmolarity than synovial fluid, but have been considered safe with no long term deleterious effects on articular cartilage or soft tissues within synovial joints (Hajek *et al.* 1990;Papacharalampous *et al.* 2005). Further, the reported adverse effects of the composition of intra-articular media on soft tissues are likely to be related to the chemical structure of the injected media rather that its osmolarity (Papacharalampous *et al.* 2005). Secondly, with the numbers available in this experiment, exposure to a hyperosmotic saline solution (0.9%, 600 mOsm) did not confer any significant protection within the middle and deep zones of human articular cartilage. However, it is the decrease in superficial zone cell death which is probably most relevant given that (1) the cell density is greatest within this region (**Table 6.1**) and, (2) loss in integrity of the superficial zone is thought to be the initiating event in degenerative disorders such as

osteoarthritis (Hunziker 2002;Squires *et al.* 2003). Finally, while this model of human cartilage injury provides a strategy for decreasing the extent of superficial zone chondrocyte death at the mechanically injured cartilage edge, the quality of eventual cartilage repair has not been evaluated. However, in a disc/ring composite model of bovine cartilage injury and repair, pharmacological inhibition of cell death at the injured cartilage edge increased the number of viable cells at the wound edge, prevented matrix loss and resulted in enhanced integrative cartilage repair (Gilbert *et al.* 2009). It would be reasonable to suggest that the significant decrease in percentage cell death at the injured cartilage edge exposed to hyperosmotic saline (0.9%, 600 mOsm) may promote lateral integration and cartilage repair. Nonetheless, it is recognised that to translate findings from an *in vitro* human cartilage system into better integrative cartilage repair, future research needs to be directed at developing *in vivo* animal models. These models may evaluate the effect of high osmolarity on soft tissues within synovial joints and facilitate quantitative assessment of cartilage injury and repair as a function of the osmolarity of joint irrigation solutions.

In conclusion, increasing the osmolarity of 0.9% saline from 285 to 600 mOsm was chondroprotective in a surgically relevant model of mechanical injury to human cartilage. These data suggest that increasing the osmolarity of joint irrigation solutions used during open and arthroscopic articular surgery may decrease chondrocyte death from surgical injury and hence, promote integrative cartilage repair.

# CHAPTER 7

# CHONDROCYTE SURVIVAL IN INJURED BOVINE ARTICULAR CARTILAGE - THE INFLUENCE OF SUBCHONDRAL BONE

## 7.1 HYPOTHESES

### Primary hypothesis

Excision of subchondral bone from bovine articular cartilage increases *in situ* chondrocyte death in explant culture.

### Secondary hypothesis

Any increase in chondrocyte death could be abrogated by co-culturing cartilage with the excised subchondral bone.

## 7.2 CHAPTER SUMMARY

The objective was to determine whether subchondral bone influences *in situ* chondrocyte survival in mechanically injured bovine articular cartilage. Injured (scalpel cut) bovine explants were cultured in serum-free media over 7 days with (1) subchondral bone excised from articular cartilage (Group A) (2) subchondral bone left attached to articular cartilage (Group B) and, (3) subchondral bone excised, but co-cultured with articular cartilage (Group C). Using CLSM, fluorescent probes and biochemical assays, *in situ* chondrocyte viability and relevant biophysical parameters (cartilage thickness, cell density and culture medium composition) were quantified over time (2.5 hours vs. 7 days). There was a significant increase in chondrocyte death over 7 days mainly within the superficial zone for Group A, but not for Groups B or C ($p<0.05$). There was no significant difference in cartilage thickness or cell density between Groups A, B and C ($p>0.05$). Increases in the protein content of the culture media for Groups B and C (but not for Group A) suggested that the release of soluble factors from subchondral bone may have influenced chondrocyte survival. In

conclusion, subchondral bone significantly influenced chondrocyte survival in articular cartilage during explant culture.

## 7.3 CHAPTER INTRODUCTION

In the experiments detailed in Chapter 3, injured bovine articular cartilage was cultured in DMEM over 7 days but there was no increase in the extent of cell death either at the injured cartilage edge or within the uninjured region of the articular surface over this time period. This finding contrasts with a number of reported studies where delayed, secondary cell death occurred in injured articular cartilage over a period of 6 hours to 7 days for instance, following trephine (Tew *et al.* 2000;Redman *et al.* 2004) or impact trauma (Chen *et al.* 2001;D'Lima *et al.* 2001a;Borazjani *et al.* 2006). The injured cartilage edge is thought to comprise areas of necrotic and apoptotic cell death, with necrosis occurring at the mechanically injured edge and a 'wave' of apoptosis extending into uninjured tissue from the injured edge (Tew *et al.* 2000).

Studies that have found such a progression of chondrocyte death over a period of days following mechanical injury (Tew *et al.* 2000;Chen *et al.* 2001;Levin *et al.* 2001;D'Lima *et al.* 2001a;Redman *et al.* 2004;Borazjani *et al.* 2006), used cartilage explants (animal and human) with subchondral bone excised immediately before or after the mechanical insult. However, when articular cartilage has been left attached to subchondral bone, no progression of chondrocyte death occurred after impact trauma (Lewis *et al.* 2003;Bush *et al.* 2005b), injurious compression (Loening *et al.* 2000) or cyclical load (Chen *et al.* 2003) over a similar time period, although none of

these studies were specifically designed to investigate whether progression of cell death in injured cartilage may be related to cartilage attachment to subchondral bone. In the experiments performed in this work so far, all explants were cultured with cartilage left attached to subchondral bone. The lack of any subsequent increase in cell death over 7 days in explant culture would support the observation that cartilage attachment to subchondral bone may be an important factor in any increase in cell death following the mechanical insult. However, the influence of cartilage attachment to subchondral bone and its effect on *in situ* chondrocyte viability has not been investigated in a controlled experiment. The primary hypothesis for this experiment was that excision of subchondral bone from articular cartilage increases *in situ* chondrocyte death in explant culture.

Articular cartilage is avascular, aneural and alymphatic. It is well established that chondrocytes interact with synovial fluid and derive various nutrients essential for their survival via diffusion (McKibbin1973). Despite the intimate anatomical association of subchondral bone to articular cartilage in synovial joints, potential interactions between subchondral bone and articular cartilage have received little attention (Mansell, Collins, & Bailey 2007). This may be because the basal calcified zone juxtaposing the osteochondral (bone-cartilage) junction has been considered an impermeable barrier to solute and fluid transport (McKibbin 1973;Clark 1990).

Our understanding of the ultrastructural features of the osteochondral junction has improved with advances in imaging modalities. ‘Gaps’ at the bone-cartilage interface have been identified which cross from subchondral bone into deeper layers of

hyaline cartilage and are visible not just with scanning electron microscopy (Redler *et al.* 1975) but also with magnetic resonance imaging (MRI) (Imhof *et al.* 1999;Imhof *et al.* 2000). In a study designed to investigate solute transport across the osteochondral junction using a novel imaging method based on fluorescence loss induced by photobleaching (FLIP), the diffusivity of sodium fluorescein (376 Da) was quantified to be $0.07 \pm 0.03$ between subchondral bone and calcified cartilage and $0.26 \pm 0.22$ $\mu m^2/s$ within the calcified cartilage in the murine distal femur (Pan *et al.* 2009). In another study, the permeability of the tidemark and calcified cartilage was investigated in plugs of equine cartilage and subchondral bone using fluorescein and rhodamine (MW ~ 400 Da) as tracers (Arkill & Winlove 2008). Imaging with quantitative fluorescence microscopy on histological sections indicated that calcified cartilage was permeable to the solutes, both from the superficial and the subchondral sides. Although these investigations suggest that gaps may provide a direct connection between bone and cartilage as shown by the passage of labelled substances between the medullary cavity and articular cartilage, evidence confirming *in vivo* physiological interactions between subchondral bone and articular cartilage is lacking, due to methodological problems with simulating the transfer of endogenous factors across the bone-cartilage interface (McKibbin 1973;Malinin & Ouellette 2000).

In the absence of evidence for the transfer of endogenous factors across the bone-cartilage interface, assessment of chondrocyte survival after removal of the subchondral bone may help establish whether subchondral bone and articular cartilage exist as a functional unit (McKibbin 1973). There are only limited data

concerning the effects of removing subchondral bone from articular cartilage *in vivo.* In non-human primates, obstruction of contact between articular cartilage and vascularised subchondral bone with methylmethacrylate (cement) resulted in cartilage degeneration within three years (Malinin *et al.* 2000). This study supports the notion that synovial fluid dependent cartilage survival is not indefinite and cartilage may rely on bone-dependent survival signals. The secondary hypothesis for this experiment was that any increase in chondrocyte death could be abrogated by co-culturing cartilage with the excised subchondral bone.

The aim of this experiment was to determine whether subchondral bone influences *in situ* chondrocyte survival in injured bovine articular cartilage. A bovine co-culture system and CLSM were used to determine chondrocyte viability in articular cartilage with or without the presence of subchondral bone in explant culture. *In situ* chondrocyte death was spatially quantified in injured articular cartilage following: (1) excision of subchondral bone from articular cartilage and, (2) co-culture of the articular cartilage with the excised subchondral bone. Biophysical parameters within the model relevant to *in situ* chondrocyte viability (cartilage thickness, cell density, culture medium composition) were also measured.

## 7.4 RESULTS

### 7.4.1 Spatial distribution of cell death

Coronal CLSM reconstructions imaging the full thickness of cartilage indicated chondrocyte death was localized near the articular surface (superficial zone) with relative sparing of the middle and deeper zones of cartilage for all explants at 2.5

hours (controls) and 7 days (**Figure 7.1**). The 'baseline' cell death in explants at 2.5 hours (controls) is due to the mechanical injury from the scalpel used to cut explants. At 7 days however, there was an increase in chondrocyte death within this injured, uppermost region of articular cartilage in Group A (bone excised) but not in Group B (bone attached) or Group C (bone co-culture).

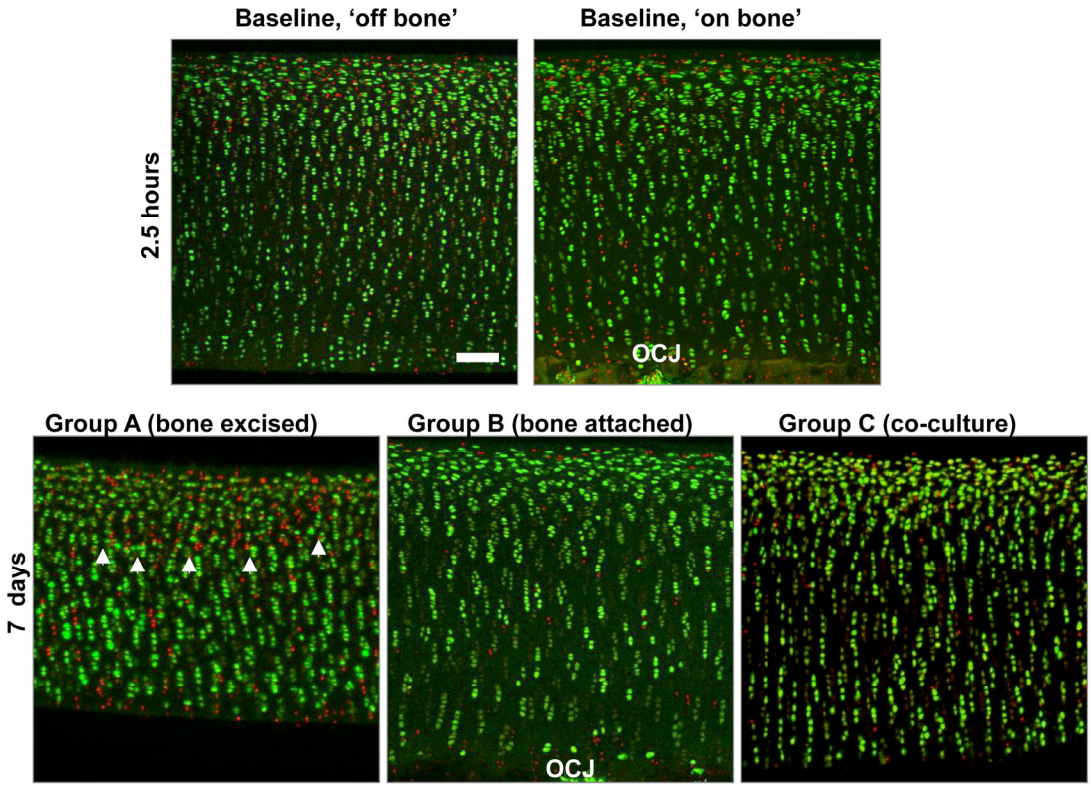


**Figure 7.1: Coronal CLSM reconstructions imaging the full thickness of cartilage**

Top panel shows the coronal CLSM reconstructions for baseline, control explants at 2.5 hours with *in situ* chondrocyte death (due to the scalpel injury) localised near the articular surface, with relative sparing of the middle and deeper zones of cartilage. Bottom panel shows the coronal CLSM reconstructions for Groups A, B and C at 7 days, with an increase in cell death within the uppermost, injured region of articular cartilage for Group A (block arrows), but not for Groups B or C (PI stains the nuclei of dead cells red; CMFDA stains the cytoplasm of live cells green, OCJ = osteochondral junction; white bar = 100μm).

Axial CLSM reconstructions imaging the superficial zone ‘top-down’ from the articular surface showed a band of *in situ* chondrocyte death at the injured cartilage edge in baseline explants ‘on’ and ‘off’ bone at 2.5 hours. This band of cell death was due to scalpel injury and equivalent to that seen in previous experiments (Chapters 3, 4 and 5). *In situ* chondrocytes in the adjacent uninjured regions of the articular surface remained viable in the baseline explants ‘on’ and ‘off’ bone at 2.5 hours. However, there was a marked increase in chondrocyte death for Group A in this uninjured region of the articular surface over 7 days. The spatial pattern of this cell death was not consistent with clear progression from regions of cartilage in which initial (baseline) cell death was caused by the scalpel injury. Instead, it was diffusely distributed throughout the entire extent of the uninjured articular surface (**Figure 7.2**). There was no such increase in chondrocyte death at the articular surface for Groups B and C at 7 days.

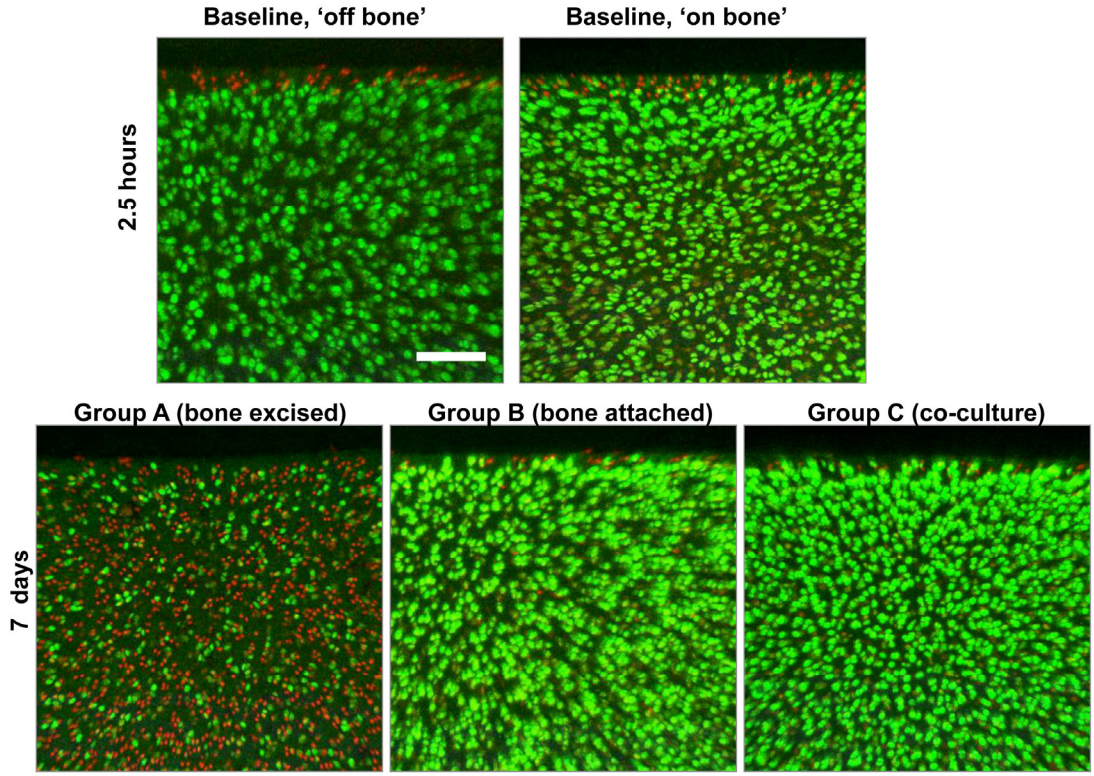


**Figure 7.2: Axial CLSM reconstructions imaging the superficial zone of cartilage 'top-down' from the articular surface**

Top panel shows the axial CLSM reconstructions for baseline, control explants at 2.5 hours with a 'band' of *in situ* chondrocyte death (due to scalpel injury) at the cut edge of the articular surface. In contrast, cells in the uninjured region of the articular surface are viable (stained green). Bottom panel shows the axial CLSM reconstructions for Groups A, B and C at 7 days. Note for Group A, the increase in chondrocyte death diffusely involving the previously uninjured region of the articular surface. In contrast, cells in this region remained viable over 7 days for Groups B and C (white bar = 100μm).

### 7.4.2 Percentage cell death

Percentage chondrocyte death quantified from coronal CLSM reconstructions ($PCD_{FT}$) confirmed cell death localized to the first 100μm from the articular surface for baseline, control explants at 2.5 hours and for explants in Groups A, B and C at 7

days (N=6, n=60, observations from two replicates from the same animal averaged for each data point, analysis of variance (ANOVA) for increasing depth from the articular surface with post-hoc Tukey's test for each depth interval, $p<0.05$ for the 0-100 μm depth-interval for each experimental group, **Figure 7.3A**). Pairwise comparisons for $PCD_{FT}$ within the 0-100 μm depth-interval indicated a significant increase from 2.5 hours to 7 days for Group A (N=6, p=0.03, compared with $PCD_{FT}$ for baseline 'off bone' controls) but not for Group B (N=6, p=0.8, compared with $PCD_{FT}$ for baseline 'on bone' controls) or Group C (N=6, p=0.6, compared with $PCD_{FT}$ for baseline 'off bone' controls).

Percentage chondrocyte death quantified from axial CLSM reconstructions ($PCD_{SZ}$) increased significantly from 2.5 hours to 7 days for Group A (N=6, n=24, observations from two replicates from the same animal averaged for each data point, compared with $PCD_{SZ}$ for baseline 'off bone' controls, paired t-test, p=0.01) but not for Group B (N=6, n=24, observations from two replicates from the same animal averaged for each data point, compared with $PCD_{SZ}$ for baseline 'on bone' controls, p=0.9) or Group C (N=6, n=24, observations from two replicates from the same animal averaged for each data point, compared with $PCD_{SZ}$ for baseline 'off bone' controls, p=0.1, **Figure 7.3B**). $PCD_{SZ}$ was similar for the baseline, control explants 'on' and 'off' bone at 2.5 hours (N=6, n=24, observations from two replicates from the same animal averaged for each data point, p=0.6), but significantly greater for Group A compared to Group B (p=0.01) or C (p=0.001) at 7 days.

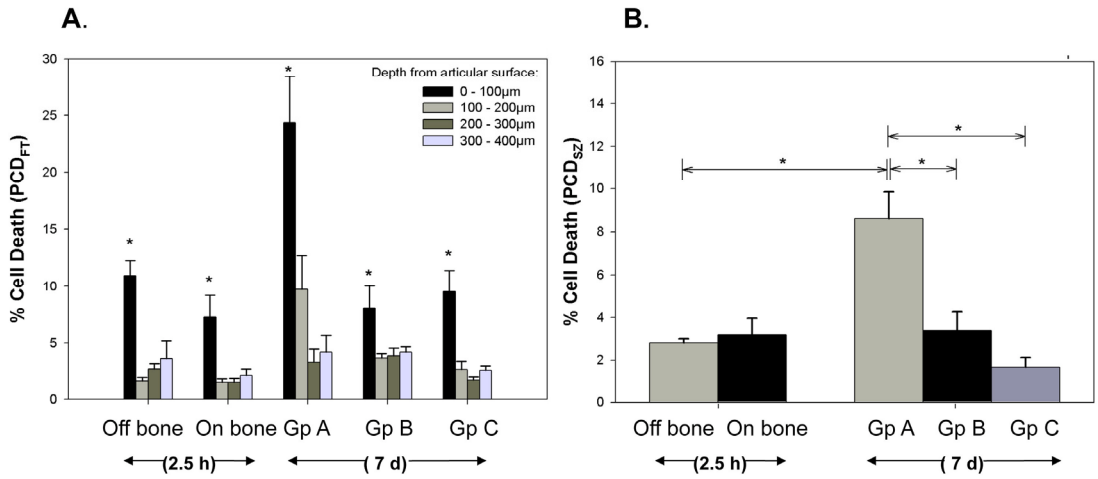


**Figure 7.3: Pooled data quantifying the increase in superficial zone chondrocyte death for Group A over 7 days**

**A. $PCD_{FT}$:** Cell death mainly occurred in the 0-100 µm depth interval (i.e. the superficial zone) for all groups (* = $p<0.05$, ANOVA for increasing depth-interval from the articular surface), but compared to baseline cell death at 2.5 hours, there was a significant increase in $PCD_{FT}$ over 7 days within the 0-100 µm depth interval for Group A but not for Groups B and C (pairwise comparisons, $p<0.05$).

**B. $PCD_{SZ}$**: There was a significant increase in cell death at the articular surface (superficial zone) from 2.5 hours to 7 days for Group A but not for Groups B and C. $PCD_{SZ}$ was also significantly greater for Group A compared to Groups B and C at 7 days (* = paired comparisons, $p<0.05$).

In summary, these data suggest an increase in superficial zone chondrocyte death with excision of subchondral bone from articular cartilage (Group A). The increase in chondrocyte death within the superficial zone was prevented if cartilage was either left attached to subchondral bone (Group B) or co-cultured with the excised subchondral bone (Group C).

### 7.4.3 Cartilage thickness and cell density

Since bovine cartilage from the metacarpophalangeal joint is very thin (< 1 mm), cartilage thickness was measured to ensure excision of subchondral bone did not result in loss of cartilage depth in explants. Coronal CLSM reconstructions of the articular cartilage were used to measure cartilage thickness as the length of a line extending from the centre of the articular surface to the cut edge (chondral explants) or osteochondral junction (osteochondral explants), ensuring accurate overlay in the x, y and z planes. The mean thickness of articular cartilage for explants in Group A, B and C was 562 ± 21 μm, 584 ± 19 μm and 516 ± 14 μm respectively, with no significant difference between groups (N=6, paired t-test, $p>0.05$ for all comparisons). For all three groups, there was no significant change in cell density within the imaged volume of cartilage from 2.5 hours to 7 days (N=6, paired t-test, $p>0.05$ for all comparisons, **Table 7.1**).

| | **Cell density at 2.5 hours (No. of cells/mm$^3$ x 10$^3$ )** | | **Cell density at 7 days (No. of cells/mm$^3$ x 10$^3$)** | | |
|---|---|---|---|---|---|
| | *Off bone* | *On bone* | *Group A* | *Group B* | *Group C* |
| $CD_{FT}$ | 47.4 ± 3.1 | 47.4 ± 4.0 | 46.9 ± 3.1 | 42.5 ± 1.8 | 46.9 ± 5.3 |
| $CD_{SZ}$ | 60.0 ± 3.7 | 64.0 ± 4.9 | 64.5 ± 2.6 | 68.7 ± 1.5 | 62.2 ± 2.9 |

**Table 7.1: Change in cell density for Groups A, B and C as a function of time**

There was no significant change in cell density (mean± standard error) from 2.5 hours to 7 days ($p>0.05$ for all paired comparisons, $CD_{FT}$ = Mean cell density from coronal CLSM reconstructions to a depth of 400μm from the articular surface representing >90% of cells within the full thickness of cartilage, $CD_{SZ}$ = Cell density within the superficial zone from axial CLSM reconstructions).

### 7.4.4 Culture medium composition

The total protein was undetectable in the culture media for baseline, control explants 'on' and 'off' bone at 2.5 hours. However, at 7 days the total protein (μg protein/mg wet tissue) was significantly higher in the culture media for Group B (N=6, paired t-test, p=0.03) and Group C (p=0.02) compared to Group A (**Figure 7.4A**). Medium osmolarity significantly increased over 7 days for Groups A (N=6, paired t-test, p=0.02), B (p=0.009) and C (p=0.005) compared with the osmolarity of the media for baseline explants at 2.5 hours, but there was no significant difference between the three groups at 7 days (p>0.05 for all paired comparisons, **Figure 7.4B**).

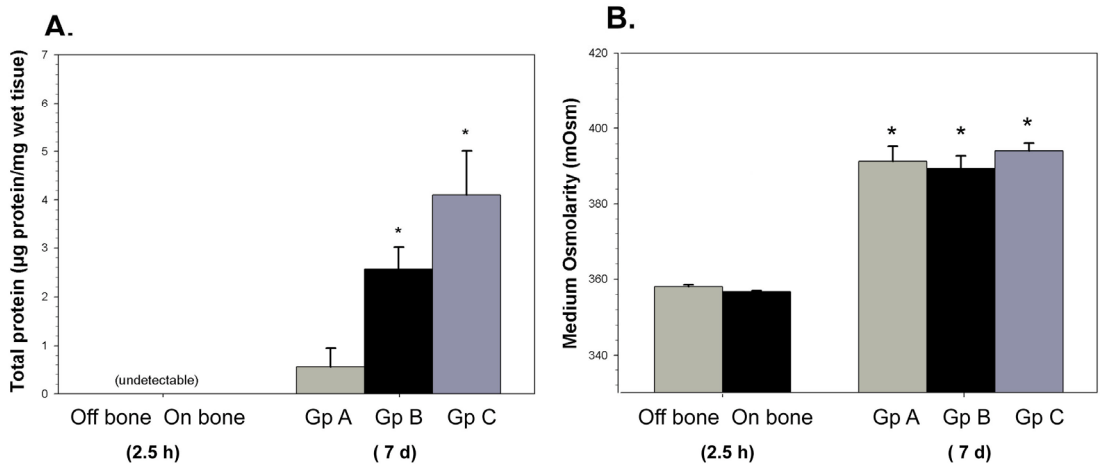


**Figure 7.4: Changes in culture medium protein content and osmolarity as a function of time**

**A:** The total protein (μg protein/mg wet tissue) was not detectable in the culture media for baseline, control explants at 2.5 hours. However, at 7 days the total protein was significantly greater in the culture media for explants in Group B and C compared to Group A (* = paired comparisons with Group A at 7 days, p<0.05).

**B:** Culture medium osmolarity significantly increased for Groups A, B and C over 7 days, but there was no difference between the groups at 7 days.

## 7.5 CHAPTER DISCUSSION

Three important conclusions can be drawn from the experiments. Firstly, excision of subchondral bone from articular cartilage resulted in an increase in chondrocyte death at 7 days that occurred mainly in the superficial zone. Secondly, the presence of subchondral bone in the culture medium abrogated this increase in superficial zone chondrocyte death. Thirdly, changes in the protein content of the culture media over 7 days would be consistent with soluble mediator(s) released from subchondral bone that may have influenced chondrocyte survival.

The use of bovine explants with scalpel injured cartilage edges identical to those used in previous experiments (Chapters 3, 4 and 5) facilitated the imaging, quantification and analyses of observed patterns of cell death as the zone of injury (scalpel cut) was standardised and reproducible. Further, with orthogonal (axial and coronal) CLSM imaging, the injured and uninjured regions of the articular cartilage were clearly distinguished allowing an assessment of the spatial pattern of *in situ* chondrocyte death in these distinct regions for the different experimental groups. Measured values of cartilage thickness were similar between explants confirming that there was no loss in cartilage depth with excision of subchondral bone from articular cartilage in Groups A and C. Cartilage cell density was similar for Groups A, B and C (**Table 7.1**) and comparable to zone-specific measurements in bovine cartilage derived using histological or stereological quantitative techniques (Wong *et al.* 1996;Stockwell & Meachim 1973). Further, cell density did not change over 7 days indicating that there were no net alterations in cartilage cellularity due to any degradative (Pennock *et al.* 2006a), apoptotic (D'Lima *et al.* 2001a) or proliferative

(van Susante *et al.* 2000) process that would have affected comparisons of percentage cell death between the three groups.

Two distinct 'patterns' of chondrocyte death could be recognized for Groups A, B and C. Firstly, there was a 'band' of superficial zone cell death at the very edge of cartilage due to the mechanical injury from the scalpel used to cut cartilage into rectangular blocks. Axial and coronal CLSM reconstructions indicated that this band of cell death increased in its extent over 7 days for Group A downwards from the articular surface but still remained localised to the upper 100 μm of articular cartilage. There was no such increase in the pattern of cell death (from scalpel injury) for Groups B and C from 2.5 hours to 7 days as indicated by their respective $PCD_{FT}$ and $PCD_{SZ}$ calculations. The second type of chondrocyte death occurred at the articular surface over 7 days after excision of subchondral bone from articular cartilage (Group A). This cell death was unrelated to the initial 'band' of cell death at the cartilage edge (from scalpel injury) and involved the entire articular surface (**Figure 7.2**). There was no clear progression of cell death from the injured edge of articular cartilage, but the pattern of increase in cell death involved the adjacent uninjured region of the articular surface. The lack any increase in cell death in this uninjured region of the articular surface if bone was left attached to articular cartilage (Group B) or co-cultured with it (Group C), further supports the observation that this increase in cell death at the articular surface was unrelated to the initial mechanical injury at the cartilage edge.

It is important to discuss the physical effects of separating cartilage from subchondral bone, as it is intuitive to attribute the additional increase in chondrocyte death at the articular surface (Group A) to the mechanical trauma involved with excising cartilage from bone. In unloaded cartilage the water content of cartilage has been reported to be affected by the osmotic pressure of the proteoglycans and the tension in the collagen network (Maroudas & Kuettner 1990). Excision of cartilage from subchondral bone will result in increased cartilage water content (and hence, cartilage swelling) as a result of the stress release in the collagen network anchored to bone (Maroudas *et al.* 1990). Further, the excision of articular cartilage (with a scalpel) may have subjected *in situ* chondrocytes to an additional mechanical insult either directly, from the scalpel injury to the deep zone or indirectly, due to shear stresses (Eberhardt, Lewis, & Keer 1991) generated immediately following release of cartilage from its osseous attachments. However, the chondroprotection with co-culture of the excised articular cartilage and subchondral bone after 7 days (Group C) indicates that chondrocyte death in Group A cannot be attributed solely to a change in physical parameters within cartilage after excision from bone. It follows that chondroprotective effects must have been exerted by subchondral bone in Group C.

There is evidence that culture of cartilage in proprietary media supplemented with fetal calf serum (FCS) rich in serum proteins and growth factors stimulates chondrocyte metabolic activity (van Susante *et al.* 2000;Redman *et al.* 2004), but it is unclear whether the addition of FCS prevents *in situ* chondrocyte death. Serum-free culture media was used in these experiments for two reasons. Firstly, the effects of endogenous bone-mediated factors that may influence chondrocyte survival could be

assessed without the potentially confounding effects of exogenous supplemental nutrient preparations. Secondly, it was evident from earlier experiments (Chapters 3 and 4) that culture of osteochondral explants in standard serum-free media did not affect *in situ* chondrocyte viability over 7 days.

Culture protein content and medium osmolarity were measured to detect any changes in the composition of the media which could have potentially influenced chondrocyte survival. The total protein content (in μg) was divided by the wet weight of the tissue present in the co-culture system to control for the variability in the size of the explants between the experimental groups. The increase in the total protein content within the culture media corresponded with a decrease in chondrocyte death for explants in Group B and C suggesting that soluble factors released from subchondral bone may have influenced chondrocyte survival. In contrast, there was no difference in medium osmolarity between the three groups at 7 days (**Figure 7.4B**). The relevance of this finding was difficult to interpret as the relative contribution of different ionic constituents to the medium osmolarity may differ between Groups A, B and C. Since the precise concentration of the ionic constituents was not determined for the co-culture system, further conclusions cannot be inferred from the data.

Explants in Groups A, B and C were incubated in a small volume (1 ml) of DMEM without any media changes over 7 days to optimise potential bone-cartilage interactions within the co-culture system. However, the exact pathway by which soluble factors (ionic or non-ionic) would pass between cartilage and bone is not known. It was likely that for explants in Group C (subchondral bone excised but co-

cultured with articular cartilage) soluble factors from bone leaked into the culture media and interacted with articular cartilage explants within the co-culture system. However, for the chondroprotective effects mediated for explants in Group B (articular cartilage left attached to subchondral bone) an alternate pathway could potentially exist via *in situ* diffusion of solutes across the bone-cartilage junction through the 'gaps' within the tidemark and calcified zone of cartilage discussed earlier (Redler *et al.* 1975;Imhof *et al.* 1999;Imhof *et al.* 2000;Arkill *et al.* 2008;Pan *et al.* 2009).

There are limitations to these experiments. Firstly, the experiments do not establish the identity of any specific chondroprotective mediators but only recognize the possibility that soluble survival factors may pass between subchondral bone and articular cartilage. A preliminary investigation to identify potential chondroprotective mediators is detailed in Chapter 10, with a list of proteins identified in the culture media used for Groups A, B and C. These proteins could represent targets for future investigation of soluble mediators involved in bone-cartilage interactions. Secondly, the methods used to identify chondrocyte death do not provide any qualitative indication regarding the mechanisms leading to cell death. However, it is likely that in the experiments presented here, cell death at the periphery of cartilage occurred due to cell necrosis in Groups A, B and C from direct scalpel injury (and did not increase with time), while the marked additional increase in cell death within the superficial zone (Group A) may have represented a form of 'non-necrotic' cell death.

In conclusion, *in vitro* experiments performed on bovine tissue confirm that excision of subchondral bone from articular cartilage resulted in an increase in chondrocyte death at 7 days that occurred almost exclusively in the superficial zone. However, the presence of subchondral bone in the culture medium prevented the increase in chondrocyte death within the superficial zone. Subchondral bone may have interacted with articular cartilage via soluble mediator(s) that influenced chondrocyte survival. Subchondral bone therefore significantly influenced chondrocyte survival in articular cartilage during explant culture. The findings from this bovine co-culture model may have relevance to the existence of a functional bone-cartilage unit within human synovial joints. The model forms the basis for the experiments performed in the next chapter investigating bone-cartilage interactions in human tissue.

# CHAPTER 8

# CHONDROCYTE SURVIVAL IN HUMAN ARTICULAR CARTILAGE - THE INFLUENCE OF HEALTHY AND SCLEROTIC SUBCHONDRAL BONE

## 8.1 HYPOTHESES

1. Excision of subchondral bone from human articular cartilage increases chondrocyte death in explant culture.
2. Co-culture of human articular cartilage with healthy subchondral bone prevents the increase in chondrocyte death.
3. Co-culture of human articular cartilage with sclerotic subchondral bone does not prevent the increase in chondrocyte death.

## 8.2 CHAPTER SUMMARY

The aim was to extend findings from the bovine co-culture model to human tissue and determine whether healthy and diseased (sclerotic) subchondral bone influences the survival of *in situ* human articular chondrocytes within a similar co-culture system. Over a period of 7 days, there was no significant increase in chondrocyte death in explant culture if subchondral bone was left attached to human articular cartilage or if the articular cartilage was excised but co-cultured with healthy subchondral bone ($p>0.05$). However, there was a significant increase in chondrocyte death over 7 days in explant culture if subchondral bone was excised from human articular cartilage or if the articular cartilage was excised but co-cultured with sclerotic subchondral bone ($p<0.05$). The extrapolation of bone-cartilage interactions *in vitro* to the clinical scenario can only be made with caution, but the findings from these experiments suggest that future investigation into *in vivo* mechanisms of articular cartilage survival and degradation, must consider cartilage interactions with subchondral bone. Further, these data suggest that bone-cartilage interactions exist in

human tissue and support the concept of a functional bone-cartilage unit within human synovial joints.

## 8.3 CHAPTER INTRODUCTION

In the bovine co-culture model detailed in the previous chapter, the spatial pattern of *in situ* chondrocyte death was defined with or without the presence of subchondral bone in a 'healthy' bone-cartilage system. Excision of subchondral bone from articular cartilage resulted in an increase in chondrocyte death that mainly occurred in the injured and uninjured regions of the superficial zone. Further, the presence of healthy subchondral bone in the co-culture system abrogated any increase in chondrocyte death. Such bone-cartilage interactions merit further investigation as they may be relevant not only to *in situ* chondrocyte viability during explant culture, but also to improving our understanding of degenerative joint diseases, such as osteoarthritis, where articular cartilage degradation co-exists with biochemical and structural alterations within the underlying subchondral bone (Radin & Rose 1986;Mansell *et al.* 2007). In the bovine experiments (Chapter 7), a 'healthy' bone-cartilage system was evaluated *in vitro* and extrapolation of findings to the clinical scenario requires caution. Nevertheless, the rapid decline in superficial zone chondrocyte viability with excision of subchondral bone from articular cartilage in bovine tissue, compares with the loss of superficial zone chondrocytes that characterizes early osteoarthritis in human cartilage (Mainil-Varlet *et al.* 2003).

Cartilage damage has generally been considered to be the initiating event in osteoarthritis, with the characteristic associated subchondral bone sclerosis thought

to be secondary. However, the concept that articular cartilage integrity may depend on the mechanical properties of its bony subchondral bed was first introduced 40 years ago (Radin, Paul, & Tolkoff 1970). It was hypothesised that the initiating event in degenerative joint disorders such as osteoarthritis may be stiffening of subchondral bone due to repetitive loading (Radin *et al.* 1970;Radin *et al.* 1986). A steep stiffness gradient subsequently develops between the basal layers of articular cartilage and subchondral bone resulting in abnormal stresses across the bone-cartilage interface and secondary loss of cartilage integrity.

More recently, biochemical alterations have been identified within subchondral bone early in the course of osteoarthritis. An evaluation of subchondral bone collagen in the femoral head of patients with osteoarthritis showed a 20-fold increase in collagen turnover, as determined by the rate of synthesis of procollagen and the rate of degradation of metalloproteinases (Bailey & Mansell 1997;Mansell, Tarlton, & Bailey 1997;Mansell & Bailey 1998;Bailey *et al.* 2004). Further analysis of the newly formed collagen suggested that the collagen fibres were narrower, with fewer cross-links and decreased mineralisation with an overall weakening of the mechanical properties of the subchondral bone (Bailey *et al.* 1997;Mansell *et al.* 1997;Mansell *et al.* 1998;Bailey *et al.* 2004).

The precise stimulus for this increased turnover of subchondral bone and whether these biochemical alterations actually precede articular cartilage degradation still remains to be determined. However, the osteoblasts present in such abnormal subchondral bone are known to undergo phenotypic alteration and produce abnormal

amounts of bone markers, proteases, growth factors, cytokines and inflammatory mediators (Bailey *et al.* 1997;Bailey *et al.* 2004;Mansell *et al.* 2007). In an *in vitro* study of osteoblast-like cell cultures prepared from the subchondral bone specimens of patients with osteoarthritis, increased levels of proteases such as urokinase plasminogen activator (uPA), insulin-like growth factor 1(IGF-1), alkaline phosphatase and osteocalcin were produced by osteoarthritic osteoblast cell cultures compared with normal cells (Hilal *et al.* 1998). The findings suggested an altered phenotype of subchondral osteoblasts that may be a contributing factor in osteoarthritis. In addition, osteoarthritic osteoblasts also directly affect cartilage metabolism as demonstrated in bone cell cultures established using explants obtained from the hip and knee joints of patients with osteoarthritis (Westacott *et al.* 1997;Westacott 2002). The conditioned media from primary osteoblasts of osteoarthritic patients significantly increased proteoglycan release from articular cartilage, a phenomenon that did not occur in media from bone cell cultures established using explants obtained from non-degenerate joints (Westacott *et al.* 1997). Finally, osteoarthritic subchondral osteoblasts co-cultured with human articular chondrocytes downregulate proteoglycan synthesis and upregulate matrix metalloproteases (MMP 3 and MMP13), factors that may further contribute to cartilage degradation (Sanchez *et al.* 2005a). Hence, while the initiating event in osteoarthritis has yet to be determined, there are an increasing number of reports that mechanical and biochemical changes in subchondral bone may play an active role in the pathogenesis of the disease and need not merely be a secondary manifestation (Mansell *et al.* 2007).

While these studies provide evidence for the integral role of changes in the metabolism of subchondral bone and bone-cartilage cross-talk during the development of osteoarthritis, the effects of healthy and diseased subchondral bone on *in situ* chondrocyte viability have not been spatially defined or quantified in human articular cartilage. The aim of this experiment was to extend findings from the bovine co-culture model to human tissue and determine whether healthy and diseased (sclerotic) subchondral bone influence the survival of *in situ* human articular chondrocytes.

Based on findings in the bovine co-culture model in the preceding chapter, it was hypothesized that (1) excision of subchondral bone from human articular cartilage increases chondrocyte death in explant culture over 7 days (2) co-culture of human articular cartilage with healthy subchondral bone prevents the increase in chondrocyte death over 7 days and, (3) co-culture of articular cartilage with sclerotic subchondral bone does not prevent the increase in chondrocyte death over 7 days. Articular cartilage explants harvested from the human femoral condyle of patients undergoing total knee replacement for osteoarthritis were placed into four groups: (1) subchondral bone left attached to articular cartilage (2) subchondral bone excised from articular cartilage (3) healthy subchondral bone excised, but co-cultured with articular cartilage and, (4) sclerotic subchondral bone co-cultured with articular cartilage. Using CLSM, *in situ* chondrocyte death has been spatially defined and quantified within the full thickness (coronal CLSM imaging) and articular surface (axial CLSM imaging) perspectives over 7 days for each of the four experimental

groups and compared with baseline cell death at 2.5 hours in articular cartilage left attached to subchondral bone.

## 8.4 RESULTS

### 8.4.1 Spatial distribution of cell death in coronal CLSM reconstructions

Coronal CLSM reconstructions of human articular cartilage visualised *in situ* chondrocytes separately in the superficial, middle and deep zone (including the osteochondral junction) of articular cartilage (**Figure 8.1**). The cell death in baseline explants at 2.5 hours represented the extent of cell death from a single scalpel cut (as coronal reconstructions imaged the cut surface of explants). Since all explants were cut with a fresh No 24 scalpel in 'push through' mode (as in previous experiments detailed in Chapters 3-7), this magnitude of cell death was assumed to be relatively constant between explants in the other experimental groups, with any differences in the observed patterns of cell death over 7 days attributable to the influence of bone in the co-culture system. The heterogeneous spatial distribution of cells was similar between explants with cells in the superficial zone arranged horizontal to the articular surface, cells in the middle zone arranged more obliquely and those in the deep zone arranged radial to the articular surface. The similarities in the spatial distribution of cells in the deep zone between explants was particularly important, as it indicated that excision of bone from articular cartilage did not result in loss of cartilage thickness and homogeneous regions of articular cartilage were imaged for subsequent quantification and comparative analyses. It was noteworthy that the superficial zone was intact in all groups confirming that these explants represented ICRS Grade 0 or early Grade 1 articular cartilage (**Figure 8.1**).

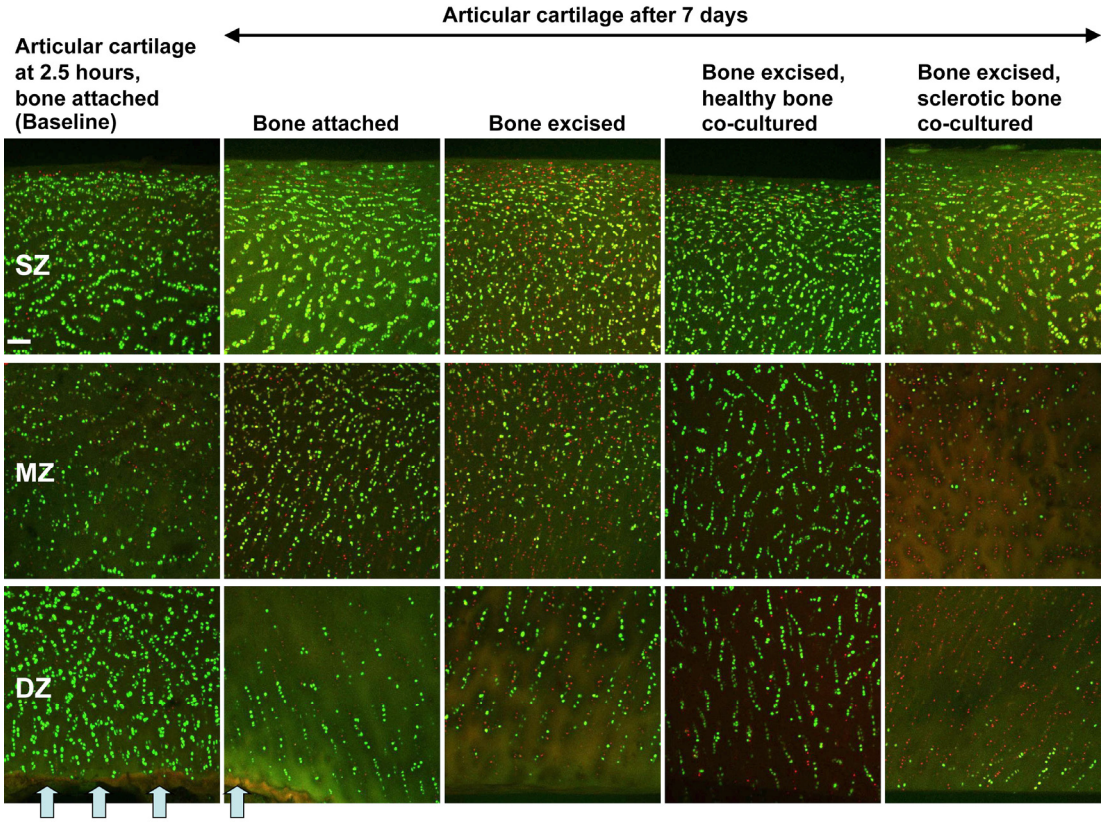


**Figure 8.1**: **Coronal CLSM reconstructions of the superficial, middle and deep zones of articular cartilage for each of the experimental groups**

Note the increased *in situ* chondrocyte death over 7 days in all zones of the articular cartilage for the panels labelled 'bone excised' and 'bone excised, sclerotic bone co-cultured'. In contrast, the majority of *in situ* chondrocytes in the panels labelled 'bone attached' and 'bone excised, healthy bone co-cultured' were viable at 7 days in keeping with the pattern of cell death observed at 'baseline' (2.5 hours). Note the osteochondral junction (block arrows) is visible on explants where bone was left attached to articular cartilage (SZ= superficial zone, MZ= middle zone, DZ=deep zone, dead cells stain red with PI, live cells stain green with CMFDA, x10 power CLSM, white bar =100 μm).

### 8.4.2 Percentage cell death in coronal CLSM reconstructions

There was no significant increase in percentage cell death over 7 days if subchondral bone was left attached to articular cartilage (N=6, n=12, two-way ANOVA, main effect for 'bone attached' versus baseline, p=0.2, **Figure 8.2**). With excision of subchondral bone from articular cartilage, there was a significant increase in percentage cell death over 7 days (N=6, n=12, main effect for 'bone excised' versus baseline, p=0.001). There was no significant increase in percentage cell death over 7 days in articular cartilage co-cultured with healthy subchondral bone (N=6, n=12, main effect for 'bone excised, healthy bone co-cultured' versus baseline, p=0.2). However, there was a significant increase in percentage cell death over 7 days in articular cartilage co-cultured with sclerotic subchondral bone (N=6, n=12, main effect for 'bone excised, sclerotic bone co-cultured' versus baseline, p=0.004).

Compared with explants in which the subchondral bone was left attached to articular cartilage over 7 days, percentage cell death was significantly greater for explants in which the bone was either excised (N=6, n=12, two way ANOVA, main effect for 'bone attached' versus 'bone excised', p=0.001, **Figure 8.2**) or sclerotic bone co-cultured with the articular cartilage (N=6, n=12, main effect for 'bone attached' versus 'bone excised, sclerotic bone co-cultured', p=0.002). However at 7 days, there was no significant difference in percentage cell death between explants in which the subchondral bone was left attached to articular cartilage and explants in which the healthy subchondral bone was excised but co-cultured with the articular cartilage (N=6, n=12, main effect for 'bone attached' versus 'bone excised, healthy bone co-cultured', p=0.6).

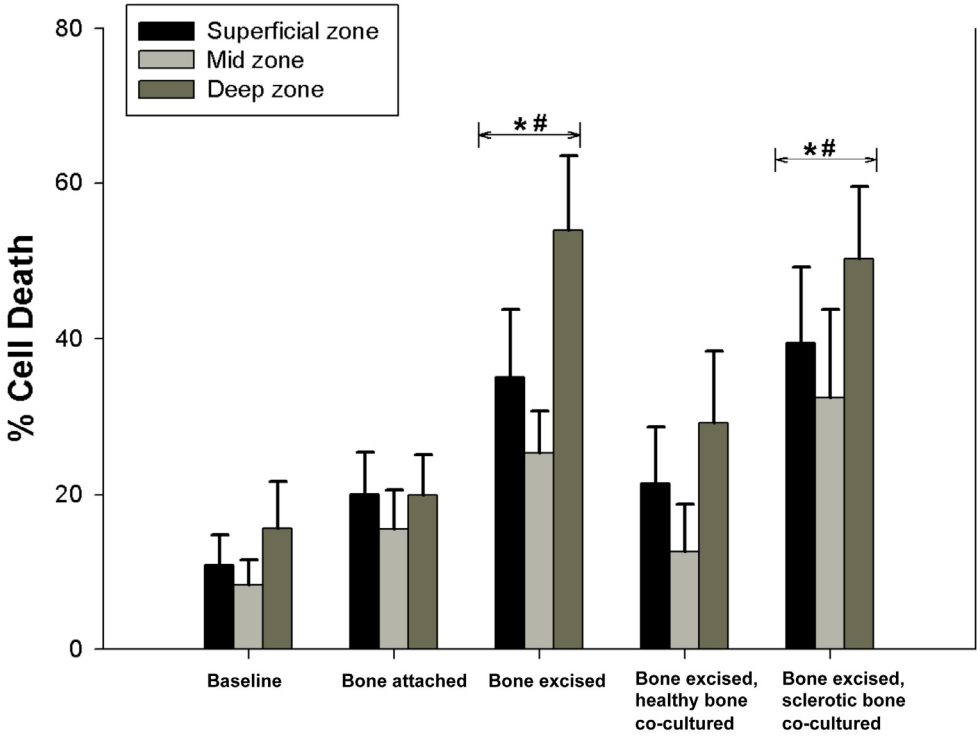


**Figure 8.2**: **Percentage cell death in the superficial, middle and deep zones of articular cartilage quantified from coronal CLSM reconstructions at 2.5 hours (baseline) and 7 days for the different experimental groups**

Compared to the percentage cell death in baseline explants at 2.5 hours, there was no significant increase in percentage cell death if subchondral bone was left attached to human articular cartilage ('bone attached') or if the articular cartilage was excised but co-cultured with healthy subchondral bone over a period of 7 days. However, there was a significant increase in percentage cell death over 7 days in explant culture if subchondral bone was excised from human articular cartilage ('bone excised') or if the articular cartilage was excised but co-cultured with sclerotic subchondral bone. At 7 days, percentage cell death was also significantly greater in these latter two groups compared to the 'bone attached' group (N=6, n=30, *$p<0.05$ ANOVA versus 'baseline', $^{\#}p<0.05$ ANOVA versus 'bone attached').

Separate comparisons (unpaired t-tests) for each zone indicated that the trend in the change in percentage cell death from 2.5 hours (baseline) to 7 days was similar in the superficial, middle and deep zones of articular cartilage (**Table 8.1**).

| | **Bone attached** | **Bone excised** | **Bone excised, healthy bone co-cultured** | **Bone excised, sclerotic bone co-cultured** |
|---|---|---|---|---|
| **SZ** | 0.2 | 0.04 | 0.2 | 0.03 |
| **MZ** | 0.3 | 0.03 | 0.6 | 0.08 |
| **DZ** | 0.6 | 0.01 | 0.3 | 0.01 |

**Table 8.1: Significance (p) values comparing percentage cell death in each of the superficial (SZ), middle (MZ) and deep (DZ) zones of articular cartilage for the four experimental groups at 7 days with baseline.**

When compared to baseline there was no significant increase in chondrocyte death over 7 days for all zones if subchondral bone was left attached to articular cartilage or if the articular cartilage was excised but co-cultured with healthy subchondral bone. However, there was a significant increase in chondrocyte death over 7 days if subchondral bone was excised from articular cartilage or if the articular cartilage was excised but co-cultured with sclerotic subchondral bone (unpaired t-tests with respective values of percentage cell death in the SZ, MZ and DZ of baseline explants).

### 8.4.3 Spatial distribution of cell death in axial CLSM reconstructions

Axial CLSM reconstructions imaged *in situ* superficial zone chondrocytes 'top-down' from the articular surface (**Figure 8.3**). In contrast to previous axial CLSM imaging of the articular surface (Chapters 3-7), the region of articular surface away from the cut edge was imaged in this experiment to evaluate cell death within the uninjured region of the articular surface. This was because in the bovine co-culture system there was no increase or progression of the band of cell death over 7 days at the injured edge of articular cartilage as quantified from the axial perspective (Chapter 7, **Figures 7.2 and 7.3B**) - cell death only occurred in the adjacent uninjured region of the articular surface over 7 days. Note that the vast majority (>97%) of superficial zone chondrocytes in this uninjured region of the articular surface are viable for the baseline explants at 2.5 hours (**Figure 8.3**). This indicated that the tissue harvested represented non-degenerate cartilage and the methods employed for harvesting the explants from discarded human osteochondral tissue did not result in any additional cell death within the uninjured region of the articular surface.

### 8.4.4 Percentage cell death in axial CLSM reconstructions

In axial CLSM reconstructions, there was no significant increase in percentage cell death over 7 days if subchondral bone was left attached to articular cartilage (N=6, n=12, 'bone attached' versus baseline, unpaired t-test, p=0.9, **Figure 8.3**). With excision of subchondral bone from articular cartilage, there was a significant increase in percentage cell death over 7 days (N=6, n=12, 'bone excised' versus baseline, p=0.01). There was no significant increase in percentage cell death over 7 days in

articular cartilage co-cultured with healthy subchondral bone (N=6, n=12, ‘bone excised, healthy bone co-cultured’ versus baseline, p=0.6). However, there was a significant increase in percentage cell death over 7 days in articular cartilage co-cultured with sclerotic subchondral bone (N=6, n=12, ‘bone excised, sclerotic bone co-cultured’ versus baseline, p=0.04).

Compared with explants in which the subchondral bone was left attached to articular cartilage over 7 days, percentage cell death was significantly greater for explants in which the bone was either excised (N=6, n=12, ‘bone attached’ versus ‘bone excised’, unpaired t-test, p=0.008, **Figure 8.3**) or sclerotic bone co-cultured with the articular cartilage (N=6, n=12, ‘bone attached’ versus ‘bone excised, sclerotic bone co-cultured, p=0.04). However at 7 days, there was no significant difference in percentage cell death between explants in which the subchondral bone was left attached to articular cartilage and explants in which the healthy subchondral bone was excised but co-cultured with the articular cartilage (N=6, n=12, ‘bone attached’ versus ‘bone excised, healthy bone co-cultured, p=0.5).

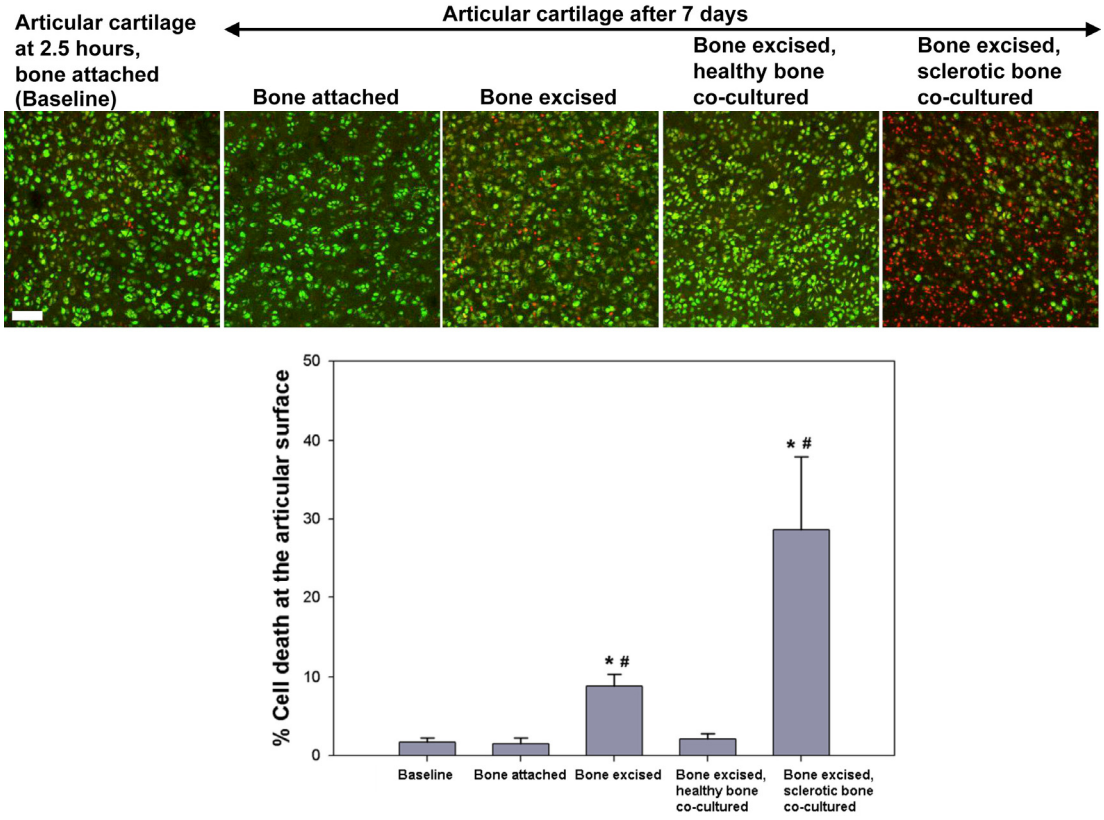


**Figure 8.3**: **Axial CLSM reconstructions of articular cartilage for each of the experimental groups with pooled data for percentage cell death**

Panel shows axial CLSM reconstructions imaging the superficial zone chondrocytes top-down from the articular surface within the uninjured region of the articular surface away from its cut edge. Note the increased *in situ* chondrocyte death over 7 days in the images labelled 'bone excised' and 'bone excised, sclerotic bone co-cultured'. In contrast, the majority of *in situ* chondrocytes in the images labelled 'bone attached' and 'bone excised, healthy bone co-cultured' are viable at 7 days in keeping with the pattern of cell death observed at 'baseline' (2.5 hours). Bar chart shows the pooled data for percentage cell death in the superficial zone of articular cartilage quantified from axial CLSM reconstructions at 2.5 hours (baseline) and 7 days and confirms these observations quantitatively (N=6, n=30, *$p<0.05$ unpaired t-test versus 'baseline', $^{\#}p<0.05$ unpaired t-test versus 'bone attached', dead cells stain red with PI, live cells stain green with CMFDA, x10 power CLSM, white bar =100 μm).

### 8.4.5 Cartilage cell density

There was no significant change in cell density calculated from coronal and axial CLSM reconstructions over 7 days for the experimental groups compared to baseline values at 2.5 hours (N=6, n=30, $p>0.05$ for all comparisons versus baseline, unpaired t-test, **Table 8.2**).

| | **Cell density (No. of cells/mm$^3$)** | | | | |
|---|---|---|---|---|---|
| | *Baseline at (2.5 hours)* | *Bone attached (7 days)* | *Bone excised (7 days)* | *Bone excised, healthy bone co-cultured (7 days)* | *Bone excised, sclerotic bone co-cultured (7 days)* |
| **$CD_{FT}$** | 8479 ± 477 | 8745 ± 1060 | 8702 ± 1082 | 8512 ± 927 | 8298 ± 1105 |
| **$CD_{SZ}$** | 27674 ± 2385 | 26533 ± 155 | 25900 ± 4255 | 24288 ± 4125 | 24728 ± 3884 |

**Table 8.2: Cell density for the each of the experimental groups**

There was no significant change in cell density (CD) between explants from 2.5 hours to 7 days ($p>0.05$ for all comparisons, unpaired t-tests compared to baseline cell density at 2.5 hours) as quantified from axial and coronal CLSM reconstructions. All data are reported as means ± standard error**.** $CD_{FT}$ = Mean cell density within the full thickness of cartilage quantified from coronal CLSM reconstructions, $CD_{SZ}$ = Cell density within the superficial zone at the articular surface quantified from axial CLSM reconstructions.

## 8.5 CHAPTER DISCUSSION

The following conclusions can be summarised from this experiment in human articular cartilage: (1) there was no significant increase in chondrocyte death over 7 days in explant culture if subchondral bone was left attached to articular cartilage or if the articular cartilage was excised but co-cultured with healthy subchondral bone and, (2) there was a significant increase in chondrocyte death over 7 days in explant culture if subchondral bone was excised from articular cartilage or if the articular cartilage was excised but co-cultured with sclerotic subchondral bone.

Ideally, only pristine articular cartilage (ICRS grade 0) would have been used for the experiments. However, as detailed in previous experiments utilising human tissue (Chapter 6, section 6.5), such pristine cartilage was not readily found from osteochondral material discarded at total knee replacement for osteoarthritis. As a compromise, early ICRS Grade 1 tissue (representing minimal fibrillation of the articular surface without loss of superficial zone cellularity) was also included in the experiment, but only after microscopic confirmation of the preservation of superficial zone cellularity using coronal and axial CLSM imaging. Of the six patients included in the study sample, ICRS Grade 0 articular cartilage was identified in four and early ICRS Grade 1 in the remaining two. The preservation of cellularity within the superficial zone was important to establish for the articular cartilage used in the experiment because the viability of the most superficial of the superficial zone chondrocytes was of particular interest in human tissue (since these cells were affected to the greatest extent in the bovine co-culture system).

The measured values of cell density from axial and coronal CLSM reconstructions (**Table 8.1**) were comparable to those reported in normal adult human articular cartilage using histological techniques (Hunziker *et al.* 2002) supporting the argument that cartilage cellularity was preserved despite the inclusion of cartilage tissue in this experiment that represented early ICRS Grade 1 degenerative changes. Further, there were no significant difference in cell density between baseline explants and explants cultured over 7 days within the experimental groups (**Table 8.1**) confirming that there were no net changes in cellularity due to apoptotic (Pennock *et al.* 2006a), degradative (D'Lima *et al.* 2001a) or proliferative (van Susante *et al.* 2000) processes during culture that may have influenced subsequent comparisons of percentage cell death.

Chondrocyte viability was preserved in all zones of articular cartilage over 7 days if subchondral bone was left attached to articular cartilage or if the articular cartilage was excised but co-cultured with healthy subchondral bone (**Table 8.1**). These findings have several implications. Firstly, the mechanical injury involved with physically separating articular cartilage from subchondral bone was not implicated in the subsequent increase in cell death observed as there was no increase in chondrocyte death with co-culture of the excised articular cartilage with healthy subchondral bone. Secondly, a biochemical (rather than a physical) interaction must have occurred between bone and cartilage that was vital for the survival of *in situ* chondrocytes within the full thickness of articular cartilage, although its precise nature remains elusive. Thirdly, the reciprocal effects of co-cultured healthy and diseased (sclerotic) bone on *in situ* chondrocyte viability add strength to the

argument that at least in explant culture, bone-cartilage interactions are an important determinant of chondrocyte survival. Finally, the use of serum-free culture media in this experiment could not have adversely affected chondrocyte viability over 7 days as cell viability was preserved in cartilage explants left attached to or co-cultured with, healthy subchondral bone in serum-free culture media. Moreover, the use of serum-free culture media may have allowed endogenous bone-cartilage interactions to occur without the potentially confounding effects of the complex mixture of exogenous nutrient factors present in proprietary serum-rich culture media.

The spatial pattern of cell death within axial CLSM reconstructions of human articular cartilage was similar to that observed in bovine tissue following excision of subchondral bone. This diffuse pattern of cell death involved the entire extent of the uninjured region of the articular surface and appeared to be unrelated to the initial mechanical injury at the cut cartilage edge. In contrast, the pattern of cell death in coronal CLSM reconstructions of injured human articular cartilage differed from bovine tissue. While in the bovine co-culture model there was an increase in cell death mainly in the superficial zone of articular cartilage following excision of subchondral bone, the increase in cell death in the human co-culture model occurred in the superficial, deep and middle zones of articular cartilage over 7 days if subchondral bone was excised from articular cartilage or if the articular cartilage was excised but co-cultured with sclerotic subchondral bone (**Table 8.1**). These differences between the patterns of cell death in animal and human models emphasise the importance of corroborating findings from animal models in human tissue. While the bovine model helped establish various parameters for a co-culture

system to evaluate bone-cartilage interactions in human tissue, the bovine model did not fully mimic human cartilage disease in keeping with the view (Roach *et al.* 1989), that extrapolation of findings from animal experiments to the human scenario requires some caution.

The opposing effects of healthy and sclerotic subchondral bone on *in situ* chondrocyte viability spatially defined and quantified in this experiment have not been reported in the literature. Several potential 'reserves' of endogenous factors important for chondrocyte survival may be implicated for the chondroprotective effects attributed to healthy subchondral bone, although evidence for their direct role in cartilage survival is still lacking. The most obvious is blood plasma, due to its similar composition to synovial fluid - a dialysate of blood plasma with the addition of certain macromolecules including hyaluronic acid (Stockwell & Meachim 1973). The bone marrow is yet another source as it contains mesenchymal stem cells capable of differentiating into adipocytic, chondrocytic and osteocytic lineages (Pittenger *et al.* 1999;Lee & Hui 2006) but evidence for the role of marrow elements in cartilage survival is lacking. Finally, bone cells may be a source of endogenous factors. It is becoming evident that cross-talk between osteoblasts and chondrocytes may play a role in cartilage degradation (Westacott *et al.* 1997;Hilal *et al.* 1998), although little is known regarding these interactions in a 'healthy' joint. With increasing subchondral sclerosis, it can be argued that the potential endogenous factors in bone important for *in situ* chondrocyte survival are no longer synthesised resulting in chondrocyte death (Grynpas *et al.* 1991;Carlson *et al.* 1996;Reboul *et al.* 2001). However, sclerotic subchondral bone has also been directly implicated in

modulating cartilage metabolism as osteoarthritic osteoblasts are known to release various cytokines and growth factors that upregulate matrix metalloproteinase synthesis and downregulate proteoglycan synthesis (Westacott *et al.* 1997;Moldovan *et al.* 1997;Reboul *et al.* 2001;Westacott 2002;Sanchez *et al.* 2005a;Sanchez *et al.* 2005b) – metabolic alterations that may eventually lead to cartilage degradation.

In the co-culture system used in this experiment, bone and cartilage explants were cultured in a small amount of media (1 ml) over 7 days without any media changes to allow potential bone-cartilage interactions to occur within the co-culture system. The decline in cell viability within human articular cartilage with excision of subchondral bone or co-culture with sclerotic subchondral bone resembled chondrocyte death that is observed in osteoarthritic articular cartilage (Mainil-Varlet *et al.* 2003) although it occurred much more rapidly (over 7 days). The reasons for this decline in chondrocyte viability over such a short period of time are not clear although the pathways of cartilage nutrition may be implicated. Since, articular cartilage is avascular, alymphatic and aneural only two other routes of cartilage nutrition exist – synovial fluid and subchondral bone. In this experiment, articular cartilage was deprived of synovial fluid during explant culture in serum-free media. With excision of subchondral bone or co-culture with sclerotic bone, articular cartilage was also deprived of the only other remaining source of cartilage nutrients. This may explain the accelerated decline in cell viability within explants cultured without subchondral bone or co-cultured with sclerotic subchondral bone. These findings support the view (Mansell *et al.* 2007) that investigation into mechanisms of cartilage degradation in osteoarthritis must consider cartilage interactions with subchondral bone. Few such

studies exist in the literature, mainly due to methodological difficulties with performing such investigation *in vivo*. In one such long term *in vivo* study, interruption of the contact between articular cartilage and subchondral bone (using cement) resulted in degeneration of the cartilage in three baboons only after 3 years, as the articular cartilage probably continued to be nourished over this period by synovial fluid (Malinin *et al.* 2000).

The findings from the experiments detailed in this chapter have certain limitations. Firstly, while bone-cartilage interactions occurred during explant co-culture, the exact pathway of such cross-talk between the two tissues remains to be established *in vivo*. Previously the calcified layer of cartilage was considered to be impermeable to solute and gases. However, more recent evidence in human cartilage indicates that gaps and channels present in the tidemark may provide pathways for the passage of nutrients from subchondral bone into articular cartilage (Redler *et al.* 1975). Further, in animal models *in situ* transport of small solutes occurs readily across the calcified layer of cartilage (Arkill *et al.* 2008;Pan *et al.* 2009). The presence of a rich vascular network in subchondral bone (Clark 1990) alongside channels crossing between the subchondral region and uncalcified cartilage (Redler *et al.* 1975;Imhof *et al.* 1999;Imhof *et al.* 2000), may facilitate the transfer of factors across the bone-cartilage interface by diffusion. Secondly, while the experiment defined the effects of bone on cartilage, the effects of cartilage (in health and disease) on bone have not been investigated. In a recent study in which chondrocytes and subchondral osteoblasts were isolated from the human knee joint but co-cultured either directly or indirectly (using conditioned media), chondrocytes from a healthy joint inhibited

osteoblast differentiation, whereas chondrocytes from an osteoarthritic joint enhanced osteoblast differentiation (Prasadam *et al.* 2010). Defining the temporal relationship of such interplay between bone and cartilage tissues would help improve our understanding of degenerative disorders such as osteoarthritis. Finally, the precise identity of the soluble factors important in bone-cartilage interactions remains to be determined. A list of potential targets from a preliminary proteomic study using conditioned media from the bovine co-culture system is detailed in Chapter 10.

In conclusion, healthy subchondral bone interacted with articular cartilage in explant culture and promoted *in situ* chondrocyte survival. Conversely, sclerotic subchondral bone was detrimental to chondrocyte viability. These data support the concept of a functional bone-cartilage unit within human synovial joints.

# CHAPTER 9

# DISCUSSION

In this section of the thesis, the discussion focuses on features of the experimental model of cartilage injury and chondroprotection developed during the course of this research. The emphasis is on aspects of the experimental model that are novel, its merits and limitations. The translational relevance of the findings to other experimental and clinical research is outlined.

## 9.1 Scalpel injured cartilage explants

The use of rectangular scalpel-injured osteochondral and chondral explants as a model to study the effect of mechanical injury on chondrocyte death has not been reported previously. The shape and size of cartilage explants harvested from animal and human tissue vary considerably in the literature. Cartilage discs are commonly harvested using a dermal or biopsy punch, sectioned using a microtome to obtain thin slices of cartilage and used for subsequent experimental work (Loening *et al.* 2000;Chen *et al.* 2001;D'Lima *et al.* 2001a;D'Lima *et al.* 2001b;Chen *et al.* 2003). Full thickness 'flakes' of cartilage may also be harvested with a scalpel (Lewis *et al.* 2003;Bush *et al.* 2005b). Since these cartilage flakes vary considerably in size and shape, more uniform coronal slices are usually produced using a microtome. Other methods of cartilage harvest have included the use of trephines (Tew *et al.* 2000;Redman *et al.* 2004) and circular osteotomes (Huntley *et al.* 2005a;Borazjani *et al.* 2006).

These instruments facilitate the sampling of cartilage but the processing of tissue into cartilage slices does incur a significant disadvantage in evaluating the responses of articular cartilage to mechanical injury. As articular cartilage is anisotropic, the

response of the tissue to a mechanical injury depends on the direction of the applied force. With sectioning into slices in the vertical plane, the cartilage explant and its response to mechanical injury can only be visualised at its sectioned (coronal) surface. While this allows assessment of the full tissue thickness, the response to the mechanical injury at the articular (synovial) surface is not visualised and is generally inferred from the pattern of injury observed in the uppermost regions of the coronal sections. When viewed in this vertical plane, superficial zone chondrocytes appear as single cells (Hunziker *et al.* 2002). However, superficial zone chondrocytes are arranged in horizontal clusters, strings, pairs as well as single cells parallel to the articular surface (Schumacher *et al.* 2002;Rolauffs *et al.* 2008). This complex arrangement of *in situ* chondrocytes at the articular surface and the ensuing pattern of cell death following the mechanical insult may not be fully appreciated with conventional sections. The articular surface - or the superficial zone – has the highest cell density within cartilage and is vital to maintaining the overall integrity of the tissue (Schumacher *et al.* 1994;Wong *et al.* 1996;Hunziker *et al.* 2002;Korhonen *et al.* 2002;Stockwell & Meachim 1973). It follows that the responses of articular chondrocytes and the extracellular matrix in the superficial zone should be of particular interest in response to any experimental mechanical cartilage injury. This region therefore merits more thorough evaluation than is feasible from thin coronal sections of cartilage.

In the model of cartilage injury established in this work, rectangular blocks of ‘on bone’ cartilage explants were produced using a scalpel to cut and create a uniform

zone of mechanical injury at the cartilage edge. Such injured cartilage explants were advantageous for several reasons:

1. The full thickness of articular cartilage was mechanically injured from the articular surface to the osteochondral junction. Since the explant was a rectangular 'block', the cartilage could be visualised from both the full thickness (coronal) and articular surface (axial) perspectives. This allowed evaluation of depth-dependent (coronal) and surface (axial) cell viability responses to mechanical injury. Further, both the injured and the uninjured regions of the articular surface were visible on the same explant allowing an assessment of the entire 'zone of injury' with visualisation of the demarcation between the viable and non-viable cells.

2. The cut edges could be orientated in the same plane, avoiding variation in matrix damage and chondrocyte death that may be attributed to the anisotropic properties of articular cartilage (Jeffery *et al.* 1991;Jeffrey *et al.* 1995). A single scalpel cut made in 'push-through' mode, without any sliding or rotating movement and the blade perpendicular to the articular surface, minimised variation in the magnitude and direction of the applied force that could potentially influence the extent of cell death between explants (Huntley *et al.* 2005a). Further, it was relatively simple to use a fresh scalpel blade for every scalpel cut preventing problems with blunting of the blade that would have otherwise confounded comparisons between explants.

3. It was important to keep medium composition (ionic and non-ionic) constant throughout incubation and culture. As scalpel trauma is associated with less chondrocyte death and matrix damage compared to blunt trauma (Tew *et al.* 2000;Redman *et al.* 2004;Huntley *et al.* 2005a), medium composition was less likely to be altered from leakage of intracellular contents (Kuhn *et al.* 2004;Bush *et al.* 2005b) and matrix components (Tew *et al.* 2000;Quinn *et al.* 2001;D'Lima *et al.* 2001b;Redman *et al.* 2004).

4. As the long cut edges of the rectangular explants were of interest for the experiment, the explants could be safely handled with the tips of forceps applied only at the short edges, avoiding any additional mechanical insult to the long edges during handling and processing of the explants which could potentially contribute to the zone of injury at the cartilage edges subsequently imaged and quantified with CLSM. Further, for the osteochondral explants used in the majority of the experiments performed here, the presence of bone underneath cartilage facilitated handling of the tissue during cartilage preparation and avoided any additional mechanical insult to the cartilage component of the explant..

5. Cartilage attachment to bone prevented curling of the specimen during incubation and culture that occurs as a result of cartilage swelling in explants that are detached from subchondral bone (Setton, Tohyama, & Mow 1998). Such explant curling of the explants would have not only problematic when handling the specimens, but the effects of swelling and alterations in the water content of the

explants were undesirable for the purposes of the experiments, especially those evaluating the effects of medium osmolarity on the extent of *in situ* chondrocyte death following mechanical injury. Additionally, following formaldehyde fixation, the bottom surface of the subchondral bone could be readily levelled with a scalpel which facilitated stable anchoring of the explant under the microscope. This minimised imaging artefact that results from tilting of the specimen during acquisition of serial optical sections with CLSM

## 9.2 Two-plane confocal laser scanning microscopy (CLSM)

The injured cartilage edges were imaged in two orthogonal planes - coronal and axial - using low-power (x10) CLSM. A series of optical sections acquired in the coronal and axial planes were then combined using imaging software to produce three dimensional reconstructions of the imaged volume of cartilage (CLSM reconstructions). This technique of two plane imaging and volume reconstructions of the injured cartilage edge was advantageous for several reasons:

1. The articular cartilage was imaged without physical sectioning which allowed imaging of the entire gross specimen with *in situ* chondrocytes visualised in three dimensions at the mechanically injured cartilage edge. This represented an advantage over conventional histology where physical sectioning of the specimen prevents the examination of the tissue in its unaltered state and poses difficulties with simultaneous observation of cell viability responses to mechanical injury. Further, with CLSM the injured zone of cartilage was visualised in its entirety from two different perspectives - the depth-dependent heterogeneity in cell death

was defined with coronal imaging and the band of cell death at the articular surface was defined with axial imaging. Such two-plane imaging was important in establishing the spatial distribution of cell death in an anisotropic tissue like cartilage as the pattern of cell death differs in the vertical (coronal) and transverse (axial) planes following mechanical injury (see section 9.1 for further details).

2. The imaged volume within CLSM reconstructions provided greater detail compared with the finer sections that are used for conventional histology. The CLSM reconstructions represent thick sections of the tissue - in bovine cartilage, the thickness of the CLSM reconstructions measured ~ 60 μm in the axial plane and ~ 80 μm in the coronal plane. In human cartilage, the thickness of the CLSM reconstruction was up to ~ 100 μm due to better penetration of the fluorescent dyes into the tissue. To illustrate this point further, a histological section (10 μm-thick) of articular cartilage stained with Mayer's haematoxylin/safranin-O is compared with a representative coronal CLSM reconstruction (60 μm-thick) from the same region (**Figure 9.1**). Note that the spatial distribution of *in situ* chondrocytes (dead and alive) and zonal heterogeneity is better appreciated in the CLSM reconstruction compared with histology due to the greater volume of tissue (and hence, greater number on cells) represented in the CLSM reconstructions.

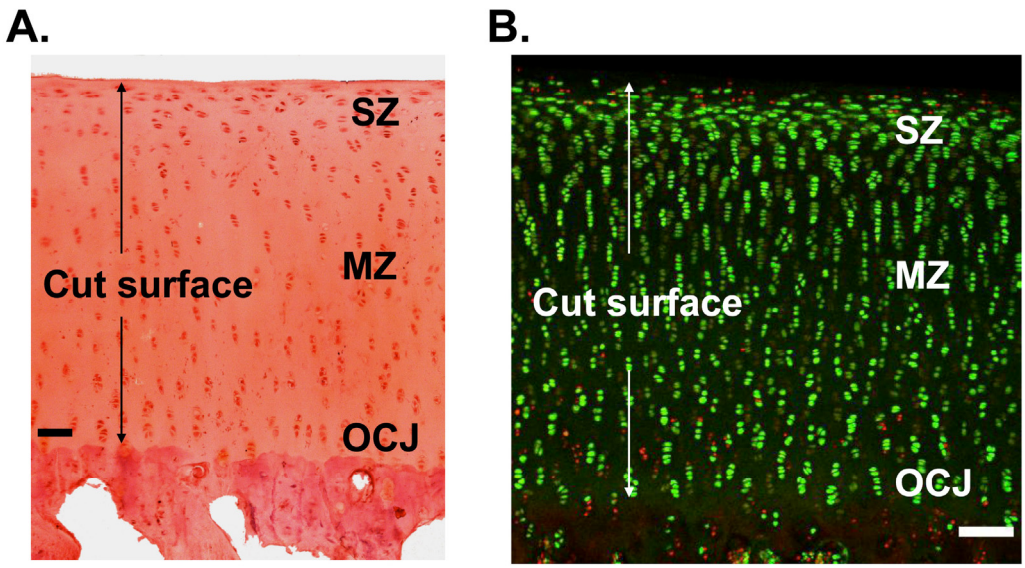


**Figure 9.1. Comparing the cut surface of articular cartilage as visualised after conventional histology and coronal CLSM reconstruction**

**A.** Low magnification (x10) photomicrograph of the cut surface of bovine articular cartilage stained with Mayer's haematoxylin and safranin-O (further detail in Figure 1.1). **B.** Low power (x10) coronal CLSM reconstruction of the cut surface of articular cartilage stained with PI and CMFDA. Note that the full thickness of cartilage is visualised in both images but due to the greater volume of the CLSM reconstruction, the cut surface is visualised in greater detail with higher cell density. (SZ=Superficial zone, MZ=mid-zone, OCJ=Osteochondral junction, black bar in **(A)** = 80µm, white bar in **(B)** = 100µm).

3. CLSM imaging of the explants from coronal and axial perspectives identified samples that needed to be excluded from the experiments due to structural abnormality or degenerative disease. Although the majority of bovine and human tissue that was degenerate could be recognised macroscopically (due to visible excoriations, grooves, surface fibrillation etc), occasionally, macroscopically normal articular cartilage was judged to be degenerate only at CLSM. For example, bovine articular cartilage from some joints was noted to be acellular in

the superficial zone with coronal CLSM imaging without any loss of structural integrity at the articular surface (**Figure 9.2**). The reasons for this pattern of loss in cartilage cellularity are not clear, but could have represented the early stages of degenerative disease. Such tissue was not considered suitable for the experiments as cell viability in the superficial zone could not be determined. Clustering of the cells at the articular surface was also indicative of degenerative disease in both bovine and human tissue (**Figure 9.3**). Such tissue was also excluded from the experiments.

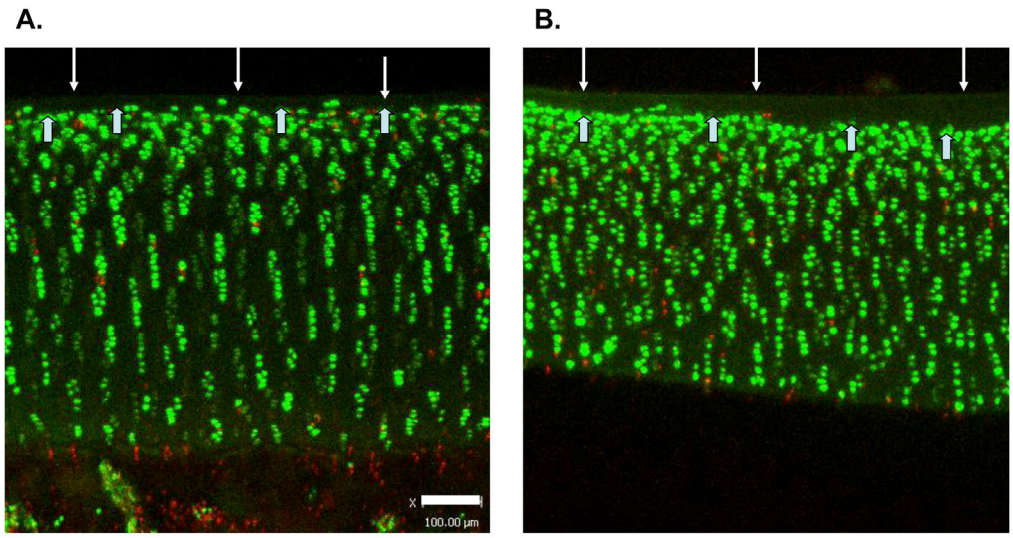


**Figure 9.2: Coronal CLSM reconstructions of bovine articular cartilage which was macroscopically normal but microscopically degenerate. (A) Explant with bone attached (B) Explant with bone excised**

Note the loss of cellularity in the superficial zone (block arrows) of articular cartilage with preservation of the integrity of the articular surface (thin arrows) in these two examples. The structure, spatial orientation and cell density of the articular chondrocytes in the deeper zones appear normal (white bar = 100µm).

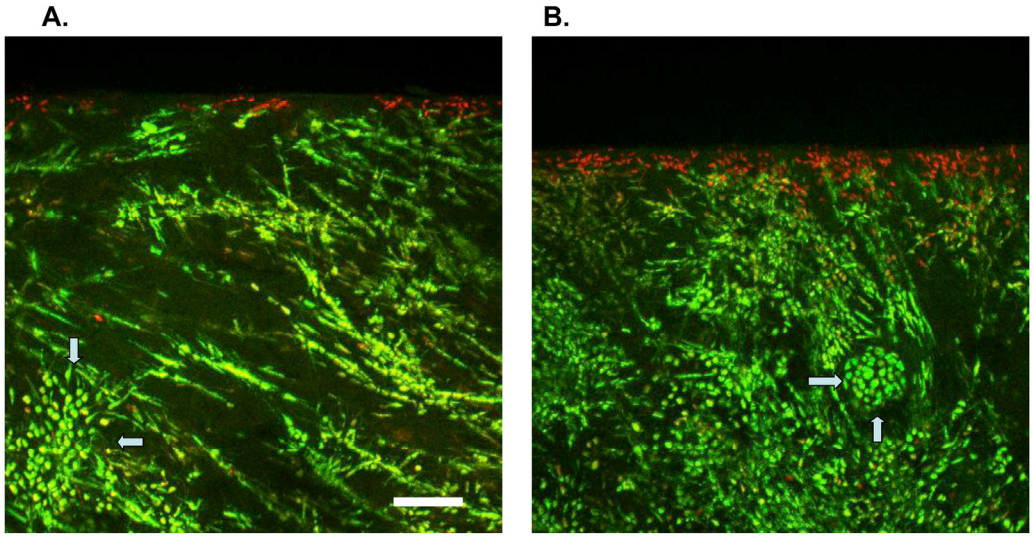


**Figure 9.3: Axial CLSM reconstructions of human articular cartilage with cell clustering at the articular surface indicative of degenerative disease**

Note the clustering of superficial zone articular chondrocytes (block arrows) in both examples. There is also significant distortion of the surface architecture, loss in cartilage cellularity and abnormal spatial distribution of the chondrocytes. These features were indicative of degenerative disease in explants and such samples were excluded from the experiments (white bar = 100μm).

4. As CLSM was used at low power (x10), relatively large areas (~ 1 x 1 $mm^2$) of the injured cartilage were imaged allowing the responses of a large number of *in situ* chondrocytes to be evaluated simultaneously. For instance, in bovine cartilage, the imaged volume of cartilage typically included over 3000 cells in the axial CLSM reconstructions and over 1000 cells in the coronal CLSM reconstructions. Subsequent quantitative analyses of cell viability based on such large numbers of cells were therefore more representative of the overall response of articular cartilage to mechanical injury and provided objective, spatial definitions of the heterogeneous, zone-specific responses of *in situ* chondrocytes to mechanical injury. Further, it was useful to be able to quickly 'scan and

screen' the entire injured cartilage edge on each explant to ensure that the zone of injury was homogenous and the optical sections acquired from the centre of the explants were representative of the explant as a whole.

5. The axial CLSM reconstructions imaged the injured and the adjacent uninjured regions of the articular surface. The temporal (2.5 hours vs. 7 days) evaluation of *in situ* chondrocyte viability in this region allowed the pattern of cell death in the two regions to be investigated with qualitative and quantitative analyses of any progression of cell death from the injured to the uninjured areas of articular cartilage. Further, the effects of altering the external environment on uninjured tissue were also evaluated within the *in vitro* system.

### 9.3 Automated quantitative image analyses

The distinct arrangement of *in situ* chondrocytes in the various regions of articular cartilage suggests a need to examine the tissue in three dimensions. Accordingly, quantitative image analyses were performed using three-dimensional image analysis software (Volocity 4.0, Improvision, Coventry, UK). Using volume reconstructions of the imaged cartilage, the software facilitated accurate determination of the size, shape and volume of individual CLSM reconstructions. Further, it helped define the spatial distribution of viable and non-viable *in situ* chondrocytes. The accuracy of positioning regions of interest (ROIs) on 'tilted' CLSM reconstructions using the image handling functionalities of the software in the x-y-z planes has already been illustrated and discussed in Chapter 2.

The software also performed automated cell viability counts within ROIs positioned on CLSM reconstructions using a validated and reproducible algorithm based on thresholding percentage voxel intensity (Jomha *et al.* 2003;Lin *et al.* 2005;Jadin *et al.* 2005). Thresholding voxel intensity minimises the inclusion of background noise from cell counts and maximises the inclusion of cells despite decreasing signal attenuation (Bush *et al.* 2001b;Jomha *et al.* 2003;Jadin *et al.* 2005). The technique proved a useful quantification tool as *in situ* chondrocytes within comparatively large ROIs, containing thousands of individual cells, were counted quickly in three-dimensions. Such automated cell counting techniques have been validated specifically for use with membrane integrity based fluorophores used to label viable and non-viable *in situ* chondrocytes (Jomha *et al.* 2003;Jadin *et al.* 2005), with a sensitivity of 99% and specificity of 98% (Jadin *et al.* 2005). While there is good correlation between automated and manual cell counts, the reproducibility of the cell counts generated by the automated algorithm is in fact significantly better compared to human evaluation (Jomha *et al.* 2003). The automated cell counts and cell density data in the experiments detailed in this thesis were comparable to those obtained using histological and stereological techniques (Wong *et al.* 1996;Hunziker 2002;Stockwell & Meachim 1973), lending support to the accuracy of the technique.

In this work, a two-fluorophore (CMFDA and PI) viability assay was used to determine *in situ* chondrocyte viability with CLSM. CMFDA labels live cells by passing freely through the cell membrane of living cells. Once inside the cell, CMFDA is cleaved by intracellular esterase and produces a membrane–impermeable product (5-chloromethylfluorescein) in the cytoplasm. PI labels non-viable cells by binding to the cellular DNA after penetrating the compromised cell membrane – as

PI is a charged molecule, it cannot pass freely through the cell membrane of viable cells. These fluorophores have been used in a number of previous studies to determine cell viability (Jones *et al.* 1985;Errington & White 1999;Poole *et al.* 2003;Chen *et al.* 2003;Huntley *et al.* 2005a;Bush *et al.* 2005b) based on the premise that cells with disrupted cell membranes are dead (and stain with PI), while those with intact cell membranes have at least retained the possibility of being viable (and stain with CMFDA).

The question then arises whether PI stained cells represent damaged cells that could potentially recover in time as these cells may have partial membrane damage that allows PI to permeate through and stain the nuclear DNA, but the cell eventually repairs the membrane damage and survives. There was no significant dye co-localisation observed in the axial and coronal images of *in situ* chondrocytes supporting the specificity of the fluorophores in determining chondrocyte viability. Further, the percentage of PI stained cells in the 'band' of superficial zone chondrocyte death at the cut cartilage edge did not decrease from 2.5 hours to 7 days for experiments performed in bovine and human articular cartilage, with cell density in cartilage remaining static over this time period. Taken together, these findings support the contention that PI stained cells represent non-viable cells that generally do not recover metabolic function over time.

Equally, it can be argued that CMFDA stained live cells may overestimate chondrocyte viability as these cells may have sustained a lethal injury that had not yet resulted in loss of membrane integrity or indeed, the cell damage may have

occurred due to intracellular events and may not have progressed to membrane disruption (McGann, Yang, & Walterson 1988) at the time the tissue is exposed to the viability stain. However, the near 100% viability of *in situ* chondrocytes in the uninjured regions of the articular surface visualised in axial CLSM reconstructions of bovine and human articular cartilage left attached to subchondral bone at 2.5 hours and 7 days, supports the notion that such false-positive identification of viable chondrocytes was unlikely to have a significant influence on measurements of *in situ* chondrocyte viability.

## 9.4 Limitations of this research

The limitations of the specific experiments have already been discussed in the relevant chapters. Some of the inherent limitations of this work applicable to the overall research strategy used to investigate *in situ* chondrocyte death after mechanical injury are discussed below.

1. While experimental mechanical injury with a scalpel is a reasonable model for predicting the responses of sharply injured articular cartilage during articular surgery (Huntley *et al.* 2005a;Huntley *et al.* 2005b;Huntley *et al.* 2005c), the nature of the mechanical injury *in vivo* during articular reconstruction is more complex and may involve a combination of blunt and sharp trauma for instance, with the use of osteotomes, suture needles, pins, intra-articular screws and motorised shavers (Hunziker 2002). The precise nature and pattern of injury to cartilage from such surgical instruments remains to be investigated. However, the osmotic sensitivity of *in situ* articular chondrocytes extends throughout all zones

of articular cartilage with a reciprocal volume response to the external medium elicited in cells within minutes (Bush *et al.* 2001b;Bush *et al.* 2005a). Therefore, it would be reasonable to suggest that the chondroprotective effects of high medium osmolarity defined in this thesis after scalpel injury may also be protective after more complex mechanical injury that occurs during articular surgery. Nonetheless, it is recognised that future experimental models of chondroprotection may need to incorporate the personality of the 'surgical injury' caused by various different surgical instruments and implants.

2. There are certain limitations with the use of CLSM to determine chondrocyte viability in whole explants following mechanical injury. Firstly, only those regions of the tissue penetrated by the fluorophores can be imaged with CLSM. This depth limitation of CLSM is dependent on the diffusion of PI and CMFDA into articular cartilage from the exposed surfaces of the explant. Deeper into the tissue, the ability of the laser light to excite the fluorophores decreases. This depth was between 60-100 μm in human and bovine tissue depending on the plane in which the cartilage was imaged. While this depth was adequate for the purposes of this work, the advent of newer imaging modalities such as two-photon laser scanning microscopy, may allow deeper imaging into the tissue than possible with CLSM (Bush *et al.* 2007). Secondly, while CLSM allows the imaging of living cells within their native environment, the focussed high intensity excitation laser light has been shown to be toxic to chondrocytes with loss of cell viability related to the duration of exposure to the excitation light (Knight *et al.* 2003). In the experiments detailed in this thesis, all explants were

fixed in formaldehyde prior to CLSM and hence, loss of cell viability from phototoxicity on the specimen was not an issue. Finally, while digital volume reconstructions (refereed to as 'CLSM reconstructions' in this thesis) overcome the problems of tissue sectioning, processing artefact and physical disturbance associated with histological assessment, they also introduce imaging artefacts from background 'noise', signal attenuation with increasing depth and non-axial image acquisition due to tilting of the specimens during microscopy. Fortunately, these problems can mostly be addressed with advances in imaging software and three-dimensional quantitative analysis.

3. The emphasis of this work has been to define *in situ* chondrocyte viability responses to mechanical injury. The extracellular matrix was not investigated in the experiments. A commonly used method for estimating the extent of matrix injury is the measurement of glycosaminoglycan release in the culture media using 1,9 –dimethylene blue as a monitor of spectrophotometric alterations that occur during the formation of the sulphated proteoglycan-dye complex (Farndale, Buttle, & Barrett 1986;Goldberg *et al.* 1993). However, the use of osteochondral explants in the majority of the experiments in this work meant that the proteoglycans would also leak from the attached subchondral bone. Due to the low specificity of the assay (Farndale *et al.* 1986) it would not have been possible to reliably distinguish proteoglycan loss in cartilage from bone.

4. Two time points were chosen to evaluate the extent of *in situ* chondrocyte death under different experimental conditions (2.5 hours vs. 7 days). The 2.5- hour time

point was chosen to reflect the duration that cartilage may be exposed to different media during open and arthroscopic surgery (Hunziker 2002). The 7-day time point was chosen to recognise secondary patterns of cell death that typically occur over this time period (D'Lima *et al.* 2001a;D'Lima *et al.* 2001b;D'Lima *et al.* 2001c;D'Lima *et al.* 2001d;Chen *et al.* 2003). By including more time points during the course of the experiments, the reproducibility of the data could have been improved. However, the limits imposed by harvesting further replicate bovine tissue samples from relatively small metacarpophalangeal joint, paucity of non-degenerate human cartilage material and the increasing overall cost of cartilage preparation and CLSM meant that a reasonable compromise had to made between the quantity of explant harvest and time course data.

## 9.5 Translational relevance to experimental and clinical research

The findings from this work are of relevance to current and future experimental and clinical research into cartilage injury, repair, degeneration and tissue engineering. Some of the most important translational aspects of this work are discussed in this section.

### 9.5.1 Fluid management systems for articular surgery

The effect of medium osmolarity on chondrocyte death in injured cartilage has important clinical implications for the design of irrigating solutions used during articular surgery. Numerous open and arthroscopic procedures involve a mechanical injury to articular cartilage, for instance during osteochondral harvest for transplantation, debridement of chondral defects or the use of intra-articular pins and

screws (Hunziker 2002). Articular cartilage has limited reparative capacity. Cartilage integrity, including chondrocyte viability, is vulnerable to mechanical injury – even that sustained during reconstructive surgery. The focal discontinuity from fibrocartilagenous repair of injured hyaline cartilage results in surface incongruity, progressive damage within native cartilage surrounding the lesion and eventual cartilage degeneration (Shapiro *et al.* 1993;Jackson *et al.* 2001;Hunziker 2002;Squires *et al.* 2003). Minimising chondrocyte death during articular surgery (by optimising the osmolarity of the irrigating fluid) would be beneficial, as it may help increase the viable cell population within the injured edge. The experiments detailed in Chapters 3 and 5 suggest that the use of hypotonic irrigating solutions such as distilled water (King, Bulstrode, & Revell 1984) could be particularly detrimental to chondrocyte viability during articular surgery. Conversely, hypertonic irrigating solutions may be chondroprotective. Indeed, chondrocyte death following mechanical injury decreased six-fold when human articular cartilage was exposed to hyperosmotic (600 mOsm) 0.9% saline (Chapter 6). These data suggest that increasing the osmolarity of joint irrigation solutions used during articular surgery may decrease chondrocyte death from surgical injury, promote integrative cartilage repair and hence, improve patient outcomes.

The early (within hours) chondroprotective effect in calcium-free media (Chapter 4) suggests that the use of joint irrigation solutions without added calcium may decrease chondrocyte death from mechanical injury during articular surgery. Joint irrigation solutions commonly used during articular surgery, such as Hartmann's solution, may contain up to 2 mM of calcium as a chloride salt. Chondrocyte death following

mechanical injury was significantly lower in media without added calcium compared to calcium-rich media at 2.5 hours, even at the lowest medium calcium concentration of 2 mM (Chapter 4). These data suggest that when choosing joint irrigation solutions, clinicians may need to decide between solutions that support chondrocyte metabolic activity (Reagan *et al.* 1983) and those that reduce chondrocyte death after mechanical injury. Irrigation solutions without added calcium, such as 0.9% Saline, may be more suitable during articular surgery as any suppression of chondrocyte metabolic activity rapidly recovers once the physiological environment is restored at the end of the surgical procedure (Arciero *et al.* 1986).

### 9.5.2 Pathogenesis of degenerative joint disease

The opposing effects of healthy and sclerotic subchondral bone on chondrocyte survival *in vitro* suggest that bone-cartilage interactions may be an important component of degenerative joint disorders such as osteoarthritis. The relevance of these latter findings to degenerative joint diseases such as osteoarthritis has already been discussed in Chapters 7 and 8. Briefly, the results support the concept of bone-cartilage cross-talk that may contribute to a functional bone-cartilage unit within synovial joints and indicate that future investigation into mechanisms of cartilage degradation in osteoarthritis must consider putative cartilage interactions with subchondral bone.

In addition to its relevance to osteoarthritis, the demonstration of biochemical bone-cartilage interactions may be important in understanding the aetiology of other diseases where structural and biochemical abnormalities in subchondral bone are

coupled to loss in integrity of the overlying articular cartilage such as osteonecrosis, osteochondritis dissecans, osteochondral fractures and bone bruising after joint instability (Zahir & Freeman 1972;Lahm *et al.* 2004;Mrosek *et al.* 2006;Uozumi *et al.* 2009). If subchondral bone proves to be an active component of the disease process contributing to articular cartilage damage for these joint disorders, it may also become a potential target for therapeutic intervention (Kwan *et al.* 2010) for slowing disease progression.

### 9.5.3 *In vitro* models of cartilage injury and repair

There are a multitude of *in vitro* animal and human models established to investigate the effects of mechanical injury on articular cartilage as mechanobiology and pathways of mechanotranduction are central to understanding cartilage injury, repair and degeneration (Hunziker 2002;Kuhn *et al.* 2004;Buckwalter, Martin, & Brown 2006). Regardless of the type of model chosen, it seems important to use cartilage explants 'on bone' if chondrocyte viability is an important aspect of the research question. Subchondral bone is often excised from articular cartilage explants during *in vitro* culture of cartilage. The technique may facilitate tissue handling and processing. In the short term (hours), separation of articular cartilage from its underlying bone is not likely to result in any increase in cell death in the uninjured regions of the cartilage explants. However, in the long term (days) – for instance, when *ex vivo* cultures are established to investigate apoptotic cell death in cartilage (D'Lima *et al.* 2001a;D'Lima *et al.* 2001d) – the cessation of bone-cartilage interactions during explant culture may cause an increase in chondrocyte death in cartilage unrelated to any other mechanical or external stimuli. Unless this 'non-

necrotic' form of cell death is accounted for by using appropriate controls, any evaluation of the extent of cell death in cartilage (Kuhn *et al.* 2004) is likely to be an overestimate.

### 9.5.4 Cartilage tissue engineering

Reconstruction of articular cartilage using tissue engineering techniques is a rapidly evolving field (Temenoff & Mikos 2000;Raghunath *et al.* 2007;Richardson *et al.* 2010). The concept of regenerating the articular cartilage surface with the growth of new cartilage is a novel approach to treating the vexing problem of articular cartilage incongruity after trauma or degeneration. The bulk of tissue engineering strategies have focused on *ex-vivo* cultivated stem-cell preparations for replacing the damaged articular surface (Temenoff *et al.* 2000;Raghunath *et al.* 2007;Richardson *et al.* 2010). The isolation, processing and culture of chondrocytes from native joints is associated with a significant mechanical insult and loss in cell viability that affects the eventual quality of regenerated tissue (Raghunath *et al.* 2007;Richardson *et al.* 2010). The chondroprotective effects of high medium osmolarity may be of relevance to optimising cell viability during *ex vivo* cultivation and improving the quality of the final regenerated tissue. Equally, the delayed (within days) increase in chondrocyte death in calcium-free media supports the use of calcium supplementation in media used during cartilage culture. Finally, the bone-cartilage interactions and in particular, the chondrocyte survival signals that may derive from healthy bone, could be relevant to newer approaches in tissue engineering that utilise the host's endogenous stem and progenitor cell population for tissue regeneration (Lee *et al.* 2010). Finally, the findings also have implications for the long term

storage of osteochondral allografts used for transplantation. Loss of chondrocyte viability has been a concern during storage of such allografts (Pennock *et al.* 2006a;Pennock *et al.* 2006b). Findings from this study support the storage of osteochondral allografts with a high bone-to-cartilage ratio (Pennock *et al.* 2006a) in order to preserve *in situ* chondrocyte viability prior to transplantation.

### 9.5.5 Routes of cartilage nutrition

Articular cartilage requires continuous nutrition in order to maintain its unique biological and mechanical properties (McKibbin 1973). Since articular cartilage is avascular, aneural and alymphatic, only two other routes of nutrition remain – through synovial fluid or the subchondral bone. *In vivo*, chondrocytes in the superficial zone rely primarily on diffusion of nutrients from synovial fluid (by virtue of their proximity to the articular surface) in order to meet nutritional requirements (Maroudas *et al.* 1968). The limiting depth of articular cartilage which can be nourished from synovial fluid is directly proportional to cartilage thickness and inversely proportional to cell density (Maroudas *et al.* 1968). Whether diffusion of nutrient solutes from synovial fluid alone is adequate in maintaining chondrocyte viability in the deep zone has not been established with certainty. Under these circumstances it has been hypothesised that deep zone articular chondrocytes may rely, at least partially, on nutrients derived via the subchondral route with transport of solutes across the osteochondral junction (McKibbin 1973). This may explain for instance, the delayed but incipient changes of matrix degradation and chondrocyte death in the deep zone of cartilage after osteonecrosis *in vivo* (Zahir *et al.* 1972) as infarction of bone must abolish any potential subchondral nourishment in the region

of cartilage overlying the dead bone. With disruption of the subchondral route of cartilage nutrition that may occur after osteonecrosis, osteochondritis dissecans or subchondral bone trauma, cartilage in the superficial zone may survive initially due to diffusion of nutrients from synovial fluid. Indeed, after physical interruption of contact (with cement) between articular cartilage and vascularised subchondral bone in primates, joint cartilage has been shown to survive for up to three years *in vivo*, presumably due to continuing nutrition from synovial fluid (Malinin *et al.* 2000). However, there is eventual loss in cartilage integrity as deep zone chondrocytes devoid of a nutrient supply from subchondral bone, and furthest removed from diffusion-dependent synovial fluid nutrition, may not be able to survive (Zahir *et al.* 1972;Malinin *et al.* 2000). While the extrapolation of *ex vivo* findings to the clinical scenario must always be made with caution, the tendency for the development of secondary degenerative changes within synovial joints as part of the natural history of untreated osteonecrosis, osteochondritis dissecans and trauma related subchondral bone lesions (Zahir *et al.* 1972;Lahm *et al.* 2004;Mrosek *et al.* 2006;Uozumi *et al.* 2009) could potentially be explained by the loss of a functional bone-cartilage unit.

## 9.6 Conclusions

A reproducible *in vitro* model of mechanically injured (scalpel cut) articular cartilage was developed in this work using bovine and human osteochondral tissue. Using fluorescence-mode CLSM, the model allowed (1) spatial and temporal quantification of *in situ* chondrocyte viability following a full thickness cartilage injury and (2) serial evaluation of three chondroprotective strategies in injured bovine and human articular cartilage: medium osmolarity, medium calcium concentration and subchondral bone attachment to articular cartilage. The conclusions from the experiments are summarised below:

1. Medium osmolarity significantly influenced superficial zone chondrocyte death in injured bovine and human articular cartilage. Greatest chondrocyte death occurred at 0 mOsm (distilled water). Conversely, a raised medium osmolarity (600 mOsm) was chondroprotective. The majority of *in situ* chondrocyte death occurred within 2.5 hours of the experimental mechanical injury, with no further increase in cell death in injured cartilage over 7 days. These data suggest that increasing the osmolarity of joint irrigation solutions used during articular surgery may decrease chondrocyte death from surgical injury.

2. Exposure of articular cartilage to calcium-free media significantly decreased superficial zone chondrocyte death in injured articular cartilage compared with exposure to calcium-rich media (2-20 mM). In calcium-rich media, the extent of chondrocyte death increased with increasing medium calcium concentration but remained localised to the superficial zone of injured articular cartilage over 7

days. However, in calcium-free media, there was an increase in chondrocyte death within deeper zones of injured articular cartilage over 7 days. The early (within hours) chondroprotective effect in calcium-free media suggested that the use of joint irrigation solutions without added calcium may decrease chondrocyte death from mechanical injury during articular surgery. The delayed (within days) increase in chondrocyte death in calcium-free media supported the use of calcium supplementation in media used during cartilage explant culture and tissue engineering.

3. Excision of subchondral bone from injured articular cartilage resulted in an increase in chondrocyte death at 7 days that occurred in the superficial zone of injured as well as the adjacent uninjured regions of articular cartilage. However, the presence of subchondral bone in the culture medium prevented this increase in chondrocyte death within the superficial zone. Subchondral bone may have interacted with articular cartilage via soluble mediator(s) that influenced chondrocyte survival. In human articular cartilage, healthy subchondral bone also interacted with articular cartilage in explant culture and promoted *in situ* chondrocyte survival, while sclerotic subchondral bone was detrimental to chondrocyte viability. The findings from these experiments suggest that future investigation into *in vivo* mechanisms of articular cartilage survival and degradation need to consider cartilage interactions with subchondral bone. Further, these data suggest that bone-cartilage interactions exist in human tissue and support the concept of a functional bone-cartilage unit within human synovial joints.

These findings are of translational relevance to fluid management systems used during open and arthroscopic articular surgery, clinical and experimental research into cartilage injury, repair and degeneration as well as current and future techniques of tissue engineering.

# CHAPTER 10

# FUTURE DIRECTIONS AND PRELIMINARY STUDIES

Several aspects of the current work have implications for further research. Some of the preliminary experiments performed to guide future research are detailed in this section.

## 10.1 Models of cartilage repair

While the *ex vivo* bovine and human models established in this work have investigated cartilage injury, repair responses were not evaluated. Several *in vitro* models of cartilage repair have been reported. Horizontal slices of cartilage have been maintained in apposition with integrative cartilage repair evaluated indirectly as a function of adhesive strength (Reindel *et al.* 1995). Another strategy involves using a full-thickness chondral explant with a central core surgically removed and re-implanted ('disc-ring composite') to study repair responses indirectly with mechanical strength testing and directly with microscopic assessment (Levin *et al.* 2001;Gilbert *et al.* 2009). While these *in vitro* models have contributed significantly to improving our understanding of integrative cartilage repair, they do require the separation of cartilage from underlying subchondral bone introducing an additional injury component that is not present in the clinical situation. Moreover, native cartilage repair occurs via fibrocartilage formation – a response intrinsically dependent on bone-cartilage interactions (Shapiro *et al.* 1993). Hence, evaluating cartilage repair responses without the influence of adjacent bone may not be representative of cartilage repair *in vivo*.

A preliminary experiment was performed to evaluate whether cartilage repair responses could be studied using the *in vitro* models of cartilage injury and bone-

cartilage co-culture developed for the purposes of this work. The methods used for the experimental mechanical injury, bone-cartilage co-culture, cell viability staining and CLSM imaging were similar to those described in this thesis with the following modification: the scalpel-injured human osteochondral explants (N=1, n=2) were cultured over a two-week period in 1 ml of serum-free DMEM without any media changes to optimise bone-cartilage interactions that may be relevant to fibrocartilagenous repair during explant culture. Coronal CLSM reconstructions of the superficial (SZ), middle (MZ) and deep (DZ) zones of the injured cartilage edges were acquired after 7 days and 14 days in culture. Interestingly, there was a significant fibroblast-like population of cells seen at the cut surface of the explant in all zones at 14 days (**Figure 10.1**). These spindle shaped cells may represent subchondral fibroblasts which have migrated to the cut surface for cartilage repair. This would be in keeping with the responses of fibrocartilagenous repair known to occur after full thickness cartilage injury from endogenous stem cell populations (Shapiro *et al.* 1993). It is possible that culturing the explant over a two week period without altering the medium allowed soluble mediators within the culture system to coordinate adherence of these cells from subchondral bone to the injured cartilage surface. The visualisation of such potential cartilage repair responses with CLSM merits investigation.

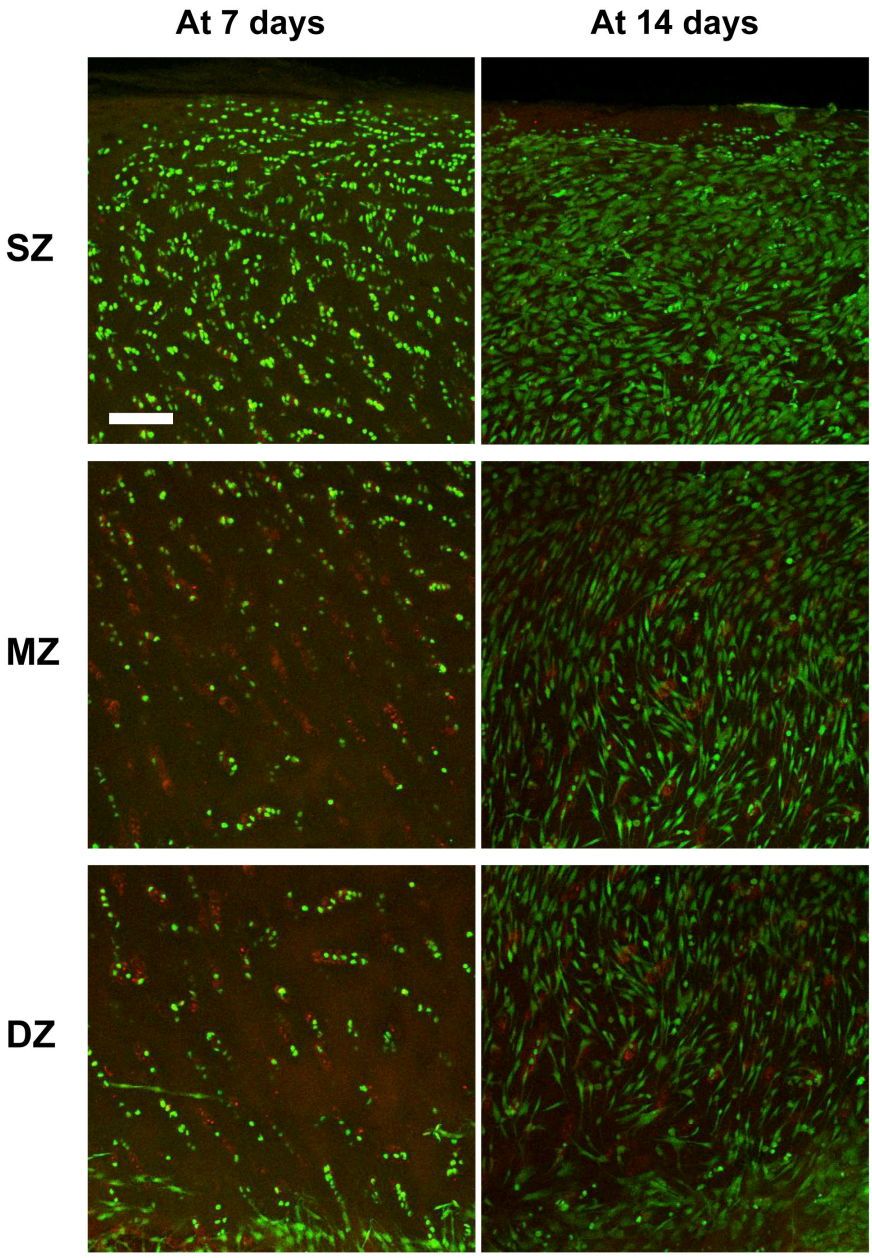


**Figure 10.1: Coronal CLSM reconstructions of an injured cartilage edge in a human osteochondral explant after 7 and 14 days in explant culture**

At 7 days, the vast majority of the *in situ* chondrocytes at the cut cartilage surface remained viable (stained green with CMFDA). There was no visible repair response although fibroblast-like cells (spindle shaped cells also stained green with CMFDA) were seen at the osteochondral junction in the DZ. At 14 days, note these fibroblast-like cells present on the entire cut surface of articular cartilage (white bar = 100 μm).

## 10.2 The influence of fetal calf serum on *in situ* chondrocyte viability

Fetal calf serum (FCS) is a complex mixture of endogenous nutrient factors and is commonly used to supplement media used for explant culture as it known to support chondrocyte metabolic and biosynthetic function (van Susante *et al.* 2000;Jochems *et al.* 2002). However, it is not known whether FCS influences *in situ* chondrocyte viability during culture.

Preliminary experiments were performed to determine whether a culture medium supplemented with FCS is capable of maintaining *in situ* chondrocyte viability and preventing the increase in chondrocyte death in the absence of subchondral bone in a co-culture system. Bovine serum albumin (BSA) is a major plasma protein in FCS (Jochems *et al.* 2002). For the experiment, rectangular blocks of bovine chondral explants were cultured over 7 days in 8 mls of DMEM supplemented with 10% FCS or 10% BSA, with explants cultured in serum-free DMEM acting as controls. All media were changed on alternate days. The cartilage explants were prepared using methods similar to those described in this work. Axial and coronal CLSM reconstructions of the articular cartilage were evaluated for the extent of *in situ* chondrocyte death at 7 days (**Figure 10.2**).

At 7 days, there was a diffuse increase in cell death in the uninjured region of the articular surface for explants cultured in serum-free DMEM and 10% BSA (top panel, **Figure 10.2)**. The pattern of this increase in cell death at the articular surface was similar to that described in Chapter 7 for cartilage explants cultured over 7 days after excision of subchondral bone (Group A explants). The percentage cell death in

this region was 32.7 ± 6.3% (mean ± standard error of mean) for explants cultured in serum-free DMEM and 20.6 ± 7.5% for explants cultured in 10% BSA with no significant difference between the two groups (N=4, n=12, paired t-test, p=0.3). The measured cell density (number of dead and live chondrocytes)/mm$^3$) in the axial CLSM reconstructions was 57.8 ± 8.7 x $10^3$ for explants cultured in serum free DMEM and 44.9 ± 5.3 x $10^3$ for explants cultured in 10% BSA, with no significant difference between the two groups (N=4, n=8, paired t-test, p=0.2). However, it was not possible to quantify percentage cell death at 7 days for the explants cultured in 10% FCS due to a significant loss in cartilage cellularity in both axial and coronal CLSM reconstructions (**Figure 10.2**). Measured cell density within the axial CLSM reconstructions for these explants was 28.9 ± 3.5 x $10^3$ – significantly lower than in the paired, control explants cultured in serum-free DMEM (N=4, n=8, paired t-test, p=0.01).

The loss of cartilage cellularity with serum-enriched DMEM represents a scientific problem in the context of its application in cell culture experiments. Since there is considerable variability between batches of FCS used to supplement culture media (Jochems *et al.* 2002;Bian *et al.* 2008) the outcome of experiments may be affected. For instance, it is likely that active serum proteases digested nuclear and cellular material from the surface of the explants over a period of 7 days in this preliminary experiment precluding subsequent quantification of cell death. The degradation of mechanical properties of bovine cartilage explants (by ~70%) in serum-supplemented media and the concurrent loss of biochemical content (30-40% proteoglycan loss) over a 2-6 week period supports the view that supplementing

explant cultures with poorly defined preparations of FCS may be detrimental to *in situ* chondrocyte viability (Bian *et al.* 2008). These preliminary experiments have important implications. Firstly, they support the use of serum-free DMEM for explant culture as the loss in cartilage cellularity is avoided. Secondly, it is unlikely that BSA is the sole player in bone-cartilage interactions important for chondrocyte survival as 10% BSA does not support *in situ* chondrocyte viability in culture. Finally, the findings indicate that future cartilage research involving FCS supplemented culture media need to utilise chemically defined or indeed, synthetic preparations of FCS to improve the 'signal to noise' ratio when determining *in situ* chondrocyte viability (Jochems *et al.* 2002;Bian *et al.* 2008).

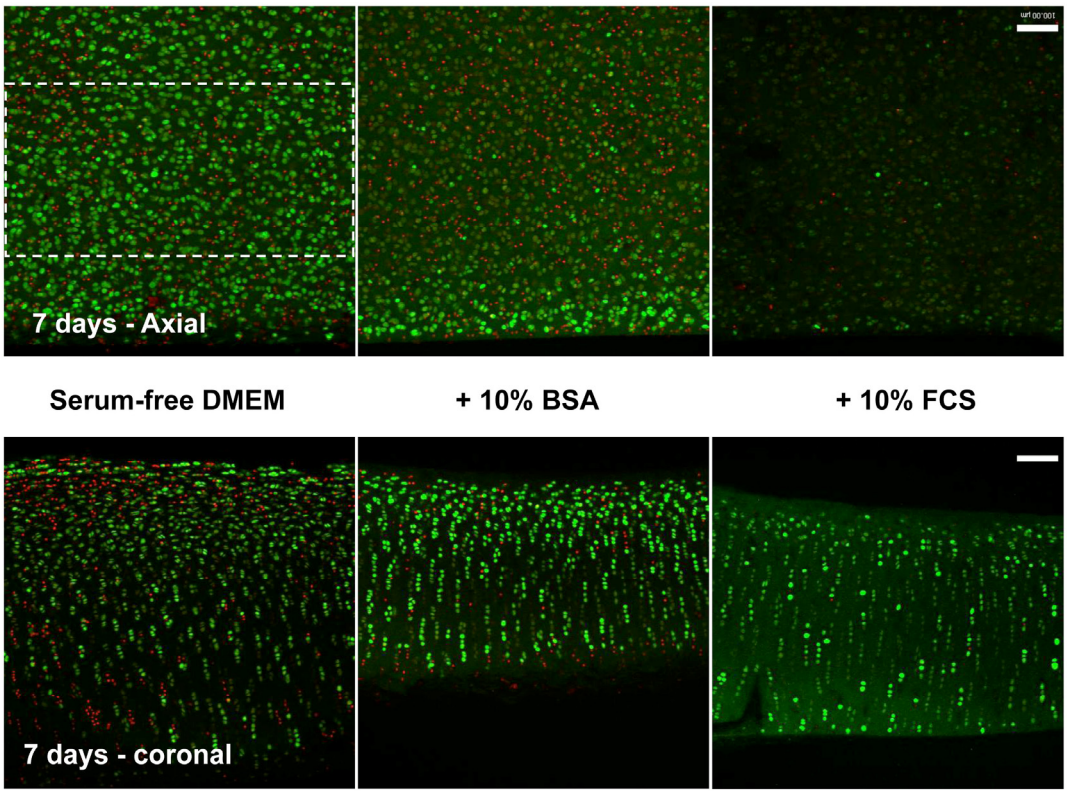


**Figure 10.2: Axial and coronal CLSM reconstruction of bovine chondral explants at 7 days cultured in serum-free DMEM, 10% BSA and 10%FCS**

Top panel shows the axial CLSM reconstructions and the bottom panel shows the coronal CLSM reconstructions of the cartilage explants. Boxed area in the top panel indicated the ROI used for quantitation of *in situ* chondrocyte death positioned on the uninjured region of the articular surface away from the zone of cell death at the cut edge of articular cartilage. Note the increase in cell death (PI labelled red cells) within this region of articular cartilage at 7 days for the explant cultured in serum-free DMEM and 10% BSA. In contrast, the axial and coronal CLSM reconstructions of the explant exposed to 10% FCS show the marked loss in cartilage cellularity. CMFDA and PI have stained the few remaining cells that are visualised within the imaged volume of cartilage exposed to FCS - the majority of surface cells have been 'lost' in culture, presumably from the effect of degradative enzymes present in FCS (white bar = 100μm).

### 10.3 Endogenous sources of chondroprotective mediators

Determining the precise nature of the chondroprotective signals within the bone-cartilage co-culture system may be of particular relevance to understanding *in vivo* mechanisms of cartilage survival and degradation. Marrow-rich blocks of healthy subchondral bone co-cultured with articular cartilage promoted *in situ* chondrocyte survival during explant culture (Chapters 7 and 8). The origin of the putative chondroprotective signals were not known and may have been derived from bone cells or marrow elements within subchondral bone.

To investigate the potential endogenous 'pools' of soluble factors that may be involved in mediating the chondroprotective effects during co-culture of healthy subchondral bone and articular cartilage, preliminary experiments were performed in bovine articular cartilage. Bovine chondral explants (off bone) were co-cultured with 'unwashed' (marrow-rich bone) and 'washed' (marrow-deplete bone) blocks of subchondral bone to determine whether bone cells influence *in situ* chondrocyte viability. 'Washed' subchondral bone was prepared by initial excision from articular cartilage and subsequent pressurised lavage of the bone block using phosphate buffered saline to remove marrow elements from the cancellous interstices. The pressurised lavage of the cancellous bone was performed using a needle and 20ml syringe. Rectangular blocks of bovine chondral explants were co-cultured in the presence of 1 ml of DMEM containing either washed or unwashed (control) blocks of subchondral bone over 7 days under identical conditions to those described for experiments detailed in Chapter 7.

The percentage cell death was quantified from axial CLSM reconstructions by positioning the ROI at a distance of 100μm from the cut cartilage edge so that cell viability was assessed solely in the uninjured region of the articular surface and the cut edge of the cartilage was not included in the measurements (as cell death occurred primarily in the uninjured region of the articular surface – see Chapter 7 for details). At 7 days, *in situ* chondrocyte viability was well preserved in articular cartilage co-cultured with washed and unwashed subchondral bone with >99% viability in the uninjured region of the articular surface (**Figure 10.3**). These preliminary findings indicated that tissue from which marrow elements had been removed still conferred protection for *in situ* chondrocytes. This suggested that potential soluble factors may arise from bone cells rather than marrow cells. Further, the data suggested that future investigation could focus on co-culturing articular cartilage in conditioned media prepared from bone cell cultures to narrow the range of soluble factors that may be key players in chondroprotective bone-cartilage interactions.

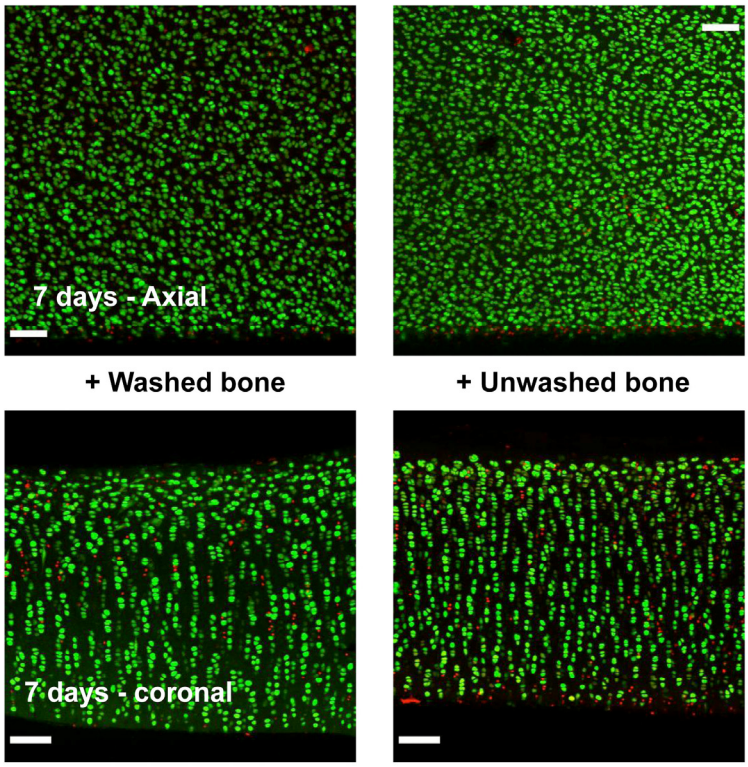


**Figure 10.3: Axial (top panel) and coronal (bottom panel) reconstructions of articular cartilage co-cultured with washed and unwashed subchondral bone at 7 days**

Note the majority (>99%) of viable *in situ* chondrocytes in the uninjured region of the articular surface in the axial CLSM reconstructions of these 'off-bone' cartilage explants (white bar = 100μm).

Similar experiments were also attempted to determine whether synovial fluid may maintain *in situ* chondrocyte viability during explant culture as it is a rich source of nutrients essential for chondrocyte survival (McKibbin 1973). Bovine synovial fluid was aspirated from the metacarpophalangeal joints of three-year old cows within 12 hours of slaughter. Serum free DMEM was supplemented with 10% synovial fluid and 'off bone' cartilage explants cultured over 7 days. However, *in situ* chondrocyte viability could not be reliably evaluated at 7 days in axial or coronal CLSM reconstructions of bovine cartilage as there was a layer of 'sludge' - including

fibroblast-like cells - that coated the surfaces of the bovine explants (**Figure 10.4**). Future research utilising CLSM to investigate the chondroprotective properties of synovial fluid may require more precise synovial fluid filtering techniques to increase the signal to noise ratio during microscopy.

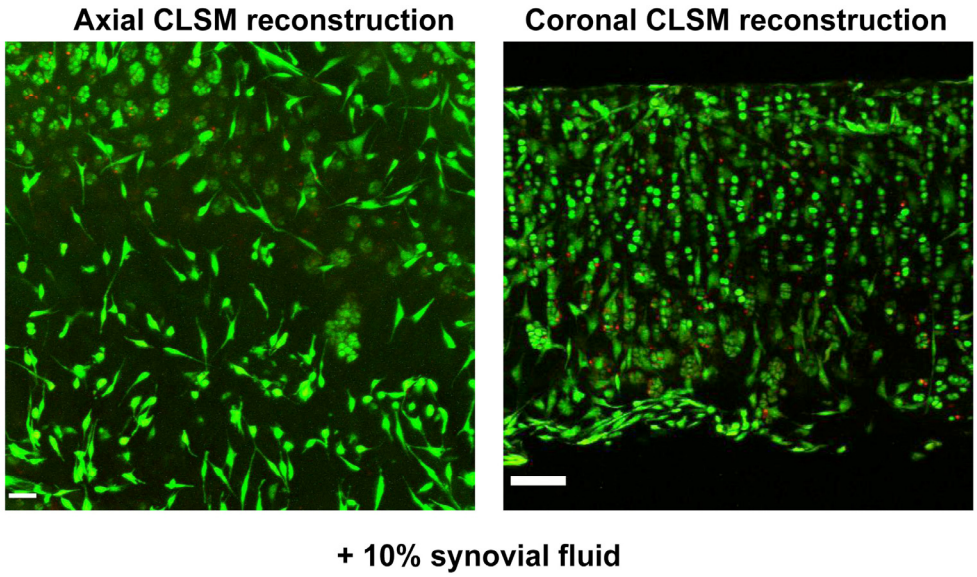


**Figure 10.4: 'Off bone' bovine cartilage explants cultured in DMEM supplemented with 10% synovial fluid**

Note the fibroblast-like cells coated over the articular surface (axial CLSM reconstruction) and cut surface (coronal CLSM reconstructions) of the bovine cartilage explants. In addition, the normal spatial distribution of the *in situ* chondrocytes was not visible due to a coating of synovial 'sludge' on the surfaces preventing adequate fluorescent dye penetration and CLSM (white bar = 100μm).

## 10.4 Proteomic studies

The precise identity of the soluble factors that may be involved in the bone-cartilage interactions remains to be established. A preliminary proteomic study of the culture media used in the bovine co-culture model was undertaken to identify soluble proteins that may play a role in bone-cartilage cross talk. The conditioned media

(N=1, n=3) was analysed at 7 days after culture of bovine cartilage explants with (1) subchondral bone excised from articular cartilage (Group A) (2) subchondral bone left attached to articular cartilage (Group B) and (3) subchondral bone excised, but co-cultured with articular cartilage (Group C). The proteomic analysis was performed in collaboration with Dr Catherine Botting at the Biomedical Sciences Mass Spectrometry and Proteomics Facility, Edinburgh and St Andrews Research School of Chemistry, St Andrews, UK. Individual proteins were identified from primary sequence databases using the Mascot search engine (www.matrixscience.com) which incorporates a scoring algorithm based on peptide mass fingerprinting (Pappin, Hojrup, & Bleasby 1993). After controlling for proteins present in DMEM, over 1000 individual proteins were identified in each of the samples of the conditioned media obtained from Groups A, B and C. A list of proteins present in the conditioned media in which chondroprotective effects were mediated (Groups B and C), but not present in the conditioned media in which chondroprotective effects were absent (Group A) is presented in **Table 10.1**. While this proteomic analysis only represents a preliminary investigation, these proteins may potentially be of interest for future research into determining molecular pathways of chondroprotective bone-cartilage interactions.

## Table 10.1: A list of proteins present in conditioned media that may play a role in chondroprotective bone-cartilage interactions in a bovine co-culture system

The proteins are listed in descending order of the Mascot score which is calculated as -10* Log(P), where P is the probability that the observed match is a random event. Mascot scores greater than 67 are significant ($p<0.05$).

| Protein ID | Protein description | Mascot Score |
|---|---|---|
| FIBB_BOVIN | fibrinogen beta chain precursor - Bos taurus (Bovine) | 1319 |
| A47391 | serum albumin precursor - rhesus macaque | 1134 |
| HBBOB | hemoglobin beta chain [validated] - bovine | 1045 |
| C3HU | complement C3 precursor [validated] - human | 745 |
| S06745 | plasminogen activator inhibitor-1 precursor - bovine | 738 |
| JN0450 | conglutinin precursor – bovine | 719 |
| AAN07165 | immunoglobulin delta heavy chain precursor - Bos taurus (Bovine) | 572 |
| CO4_BOVIN | complement C4 precursor | 565 |
| HABO | hemoglobin alpha chain [validated] - bovine | 507 |
| I59611 | antithrombin III - mouse | 500 |
| Q1KYZ5_AMMLE | beta globin chain.- Ammotragus lervia (Barbary sheep) (Aoudad). | 478 |
| HBSHBC | hemoglobin beta C(NA) chain - aoudad (tentative sequence) | 459 |
| HBHO | hemoglobin beta chain [validated] - horse | 440 |
| HBB_PIG | hemoglobin subunit beta (Beta-globin).- Sus scrofa (Pig). | 398 |
| 1VWTA | hemoglobin alpha chain mutant (V96W) (aquomet), chain A - human | 396 |
| KUHU | ferroxidase (EC 1.16.3.1) precursor [validated] - human | 396 |
| HBB_CALTO | hemoglobin subunit (Beta-globin).- Callicebus torquatus (Collared titi) | 396 |
| NBBO | apolipoprotein H precursor - bovine | 369 |
| AAC48761 | IGG3 heavy chain constant fragment - Bos taurus (Bovine) | 360 |
| A25359 | hemoglobin alpha chain - European suslik | 356 |
| Q76HN9_TAMSI | alpha1-antitrypsin-like protein - Tamias sibiricus (Siberian chipmunk) | 344 |
| HBLEF | hemoglobin beta chain - brown lemur (tentative sequence) | 331 |
| AAB66578 | immunoglobulin light chain variable region (fragment) - Bovine | 325 |
| LPRB1Z | apolipoprotein A-I precursor (clone 2Zap AI) - rabbit | 317 |
| HAGW | hemoglobin alpha chain - guanaco | 301 |
| Q76HP1_TAMSI | alpha1-antitrypsin-like protein - Tamias sibiricus (Siberian chipmunk) | 295 |
| HBOL | hemoglobin beta chain - Ehrenberg's mole-rat | 288 |
| S66290 | alpha 1 antichymotrypsin - bovine (fragment) | 280 |
| AAB03276 | IgM (Fragment) - Bos taurus (Bovine) | 279 |
| AAB68840 | immunoglobulin variable region (fragment) - Bos taurus (Bovine). | 275 |
| HAHY | hemoglobin alpha chain - golden hamster | 259 |
| Q4TWB7_HUMAN | beta globin chain (Fragment) - Homo sapiens (Human) | 255 |
| AAB01178 | immunoglobin heavy chain - Bos taurus (Bovine) | 255 |
| AAI02942 | BC102941 NID - Bos taurus | 248 |
| Q5J801_BOVIN | endopin 2B - Bos taurus (Bovine) | 235 |
| Q5ND38_MOUSE | serine proteinase inhibitor, clade F, member 1- Mus musculus (Mouse) | 231 |
| Q3T9E3_MOUSE | activated spleen cDNA, RIKEN full-length enriched library | 223 |
| MFGM_BOVIN | lactadherin precursor (Milk fat globule-EGF factor 8) (MFG-E8) | 223 |
| PNPH_BOVIN | purine nucleoside phosphorylase (EC 2.4.2.1) - Bos taurus (Bovine) | 221 |
| O97658_BOVIN | serine protease (Fragment)- Bos taurus (Bovine) | 202 |

| | | |
|---|---|---|
| G6PI_BOVIN | glucose-6-phosphate isomerase (EC 5.3.1.9) (GPI) | 202 |
| Q3TEE8_MOUSE | 2 days neonate thymus thymic cells cDNA | 192 |
| HSHUA1 | histone H2A.1 - human | 191 |
| BAB41156 | AB060222 NID - Macaca fascicularis | 190 |
| S13495 | pregnancy zone protein - human | 188 |
| JQ1984 | H3.3 like histone MH321 - mouse | 182 |
| Q3UEM7_MOUSE | adult male liver tumor cDNA, RIKEN full-length enriched library | 176 |
| Q543K9_MOUSE | adult male hippocampus cDNA | 166 |
| AAA69559 | ECU28947 NID - Equus caballus | 163 |
| A29952 | alpha-1 proteinase inhibitor III precursor - rat | 159 |
| AAU03134 | AY588268 NID - Pongo pygmaeus | 153 |
| PDIA3_PAPHA | protein disulfide-isomerase A3 (EC 5.3.4.1) (Fragments) | 153 |
| Q17QM7_BOVIN | VIM protein - Bos taurus (Bovine) | 148 |
| BAA25171 | AB008683 NID - Bos taurus | 146 |
| Q3UBP6_MOUSE | bone marrow macrophage cDNA, RIKEN full-length enriched library | 145 |
| Q3B7N2_BOVIN | hypothetical protein MGC128689 - Bos taurus (Bovine) | 145 |
| ENOA_BOVIN | alpha-enolase (EC 4.2.1.11) (2-phospho-D-glycerate hydro-lyase) | 144 |
| AAX46682 | BT021835 NID - Bos taurus | 143 |
| AAA51995 | HUMCG1PA1 NID - Homo sapiens | 140 |
| AAD23578 | AF125537 NID - Sus scrofa | 138 |
| A35685 | metalloproteinase inhibitor 1 precursor - bovine | 137 |
| Q2TU83_HUMAN | proliferation-inducing protein 35 - Homo sapiens (Human). | 134 |
| ATRB | actin, skeletal muscle - rabbit | 133 |
| ENOA_MOUSE | alpha-enolase (EC 4.2.1.11) (2-phospho-D-glycerate hydro-lyase) | 131 |
| JC2385 | protein disulfide-isomerase (EC 5.3.4.1) ER60 precursor - bovine | 131 |
| Q29574_PIG | histone H2B (Fragment) - Sus scrofa (Pig) | 126 |
| E968303 | gelosin - Homo sapiens (Human). | 123 |
| Q2KIF2_BOVIN | leucine-rich alpha-2-glycoprotein 1 - Bos taurus (Bovine). | 118 |
| AAB92652 | AF038127 NID - Equus caballus | 117 |
| AAI02334 | BC102333 NID - Bos taurus | 117 |
| H2B1B_MOUSE | histone H2B type 1-B (h2B-143) - Mus musculus (Mouse). | 114 |
| AAP41220 | AY291312 NID - Bos taurus | 109 |
| MDHC_BOVIN | malate dehydrogenase, cytoplasmic (EC 1.1.1.37) | 105 |
| FAHUA2 | alpha-actinin 2 - human | 104 |
| Q59IP3_PIG | procollagen alpha 1(V) precursor - Sus scrofa (Pig). | 103 |
| HSHUB1 | histone H2B.1 - human | 102 |
| Q7Z474_HUMAN | PSMA4 protein - Homo sapiens (Human). | 100 |
| Q91VL4_MOUSE | Col1a2 protein (Fragment).- Mus musculus (Mouse). | 98 |
| AAI05536 | BC105535 NID - Bos taurus | 96 |
| AAL13308 | AF417637 NID - Bos taurus | 96 |
| A35714 | fetuin precursor - bovine | 93 |
| Q3MHK9_BOVIN | fascin homolog 1, actin-bundling protein.- Bos taurus (Bovine). | 93 |
| CAH93278 | CR861207 NID - Pongo pygmaeus | 90 |
| Q5TCQ0_HUMAN | thioredoxin domain containing 5 - Homo sapiens (Human). | 89 |
| AAZ81421 | DQ156119S2 NID - Bos taurus | 88 |
| Q6EEI0_PIG | vimentin (Fragment) - Sus scrofa (Pig). | 87 |
| Q9R146_CAVPO | alpha actin (Fragment) - Cavia porcellus (Guinea pig). | 86 |
| VINC_HUMAN | vinculin (Metavinculin) - Homo sapiens (Human). | 83 |
| EF2_BOVIN | elongation factor 2 (EF-2) - Bos taurus (Bovine). | 80 |
| ANXA1_HORSE | annexin A1 (Annexin I) (Lipocortin I) (Calpactin II) | 79 |
| Q2KIT0_BOVIN | similar to collagen, type X, alpha 1 - Bos taurus (Bovine). | 79 |
| 1QQ2A | thioredoxin peroxidase 2 mutant | 76 |
| PGBM_HUMAN | heparan sulfate proteoglycan core protein precursor (HSPG) | 75 |
| CILP2_HUMAN | cartilage intermediate layer protein 2 precursor (CILP-2) | 75 |
| KCHUS2 | stromelysin 2 (EC 3.4.24.22) precursor [validated] - human | 74 |

## BIBLIOGRAPHY


1. Archer,C.W. & Francis-West,P. (2003) The chondrocyte. *Int.J Biochem.Cell Biol.* **35**, 401-404.

2. Arciero,R.A., Little,J.S., Liebenberg,S.P., & Parr,T.J. (1986) Irrigating solutions used in arthroscopy and their effect on articular cartilage. An in vivo study. *Orthopedics* **9**, 1511-1515.

3. Arkill,K.P. & Winlove,C.P. (2008) Solute transport in the deep and calcified zones of articular cartilage. *Osteoarthritis.Cartilage* **16**, 708-714.

4. Bailey,A.J. & Mansell,J.P. (1997) Do subchondral bone changes exacerbate or precede articular cartilage destruction in osteoarthritis of the elderly? *Gerontology* **43**, 296-304.

5. Bailey,A.J., Mansell,J.P., Sims,T.J., & Banse,X. (2004) Biochemical and mechanical properties of subchondral bone in osteoarthritis. *Biorheology* **41**, 349-358.

6. Bank,R.A., Soudry,M., Maroudas,A., Mizrahi,J., & TeKoppele,J.M. (2000) The increased swelling and instantaneous deformation of osteoarthritic cartilage is highly correlated with collagen degradation. *Arthritis Rheum.* **43**, 2202-2210.

7. Baumgarten,M., Bloebaum,R.D., Ross,S.D., Campbell,P., & Sarmiento,A. (1985) Normal human synovial fluid: osmolality and exercise-induced changes. *J.Bone Joint Surg.Am.* **67**, 1336-1339.

8. Bian,L., Lima,E.G., Angione,S.L., Ng,K.W., Williams,D.Y., Xu,D., Stoker,A.M., Cook,J.L., Ateshian,G.A., & Hung,C.T. (2008) Mechanical and biochemical characterization of cartilage explants in serum-free culture. *J Biomech.* **41**, 1153-1159.

9. Borazjani,B.H., Chen,A.C., Bae,W.C., Patil,S., Sah,R.L., Firestein,G.S., & Bugbee,W.D. (2006) Effect of impact on chondrocyte viability during insertion of human osteochondral grafts. *J.Bone Joint Surg.Am.* **88**, 1934-1943.

10. Bradford,M.M. (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal.Biochem.* **72**, 248-254.

11. Brocklehurst,R., Bayliss,M.T., Maroudas,A., Coysh,H.L., Freeman,M.A., Revell,P.A., & Ali,S.Y. (1984) The composition of normal and osteoarthritic articular cartilage from human knee joints. With special reference to unicompartmental replacement and osteotomy of the knee. *J.Bone Joint Surg.Am.* **66**, 95-106.

12. Browning,J.A., Saunders,K., Urban,J.P., & Wilkins,R.J. (2004) The influence and interactions of hydrostatic and osmotic pressures on the intracellular milieu of chondrocytes. *Biorheology* **41**, 299-308.

13. Buckwalter,J.A. & Mankin,H.J. (1998a) Articular cartilage: degeneration and osteoarthritis, repair, regeneration, and transplantation. *Instr.Course Lect.* **47**, 487-504.

14. Buckwalter,J.A. & Mankin,H.J. (1998b) Articular cartilage: tissue design and chondrocyte-matrix interactions. *Instr.Course Lect.* **47**, 477-486.

15. Buckwalter,J.A., Martin,J.A., & Brown,T.D. (2006) Perspectives on chondrocyte mechanobiology and osteoarthritis. *Biorheology* **43**, 603-609.

16. Bulstra,S.K., Kuijer,R., Eerdmans,P., & van der Linden,A.J. (1994) The effect in vitro of irrigating solutions on intact rat articular cartilage. *J.Bone Joint Surg.Br.* **76**, 468-470.

17. Bush,P.G. & Hall,A.C. (2001a) Regulatory volume decrease (RVD) by isolated and in situ bovine articular chondrocytes. *J.Cell Physiol* **187**, 304-314.

18. Bush,P.G. & Hall,A.C. (2001b) The osmotic sensitivity of isolated and in situ bovine articular chondrocytes. *J.Orthop.Res.* **19**, 768-778.

19. Bush,P.G. & Hall,A.C. (2005a) Passive osmotic properties of in situ human articular chondrocytes within non-degenerate and degenerate cartilage. *J.Cell Physiol* **204**, 309-319.

20. Bush,P.G., Hodkinson,P.D., Hamilton,G.L., & Hall,A.C. (2005b) Viability and volume of in situ bovine articular chondrocytes-changes following a single impact and effects of medium osmolarity. *Osteoarthritis.Cartilage.* **13**, 54-65.

21. Bush,P.G., Pritchard,M., Loqman,M.Y., Damron,T.A., & Hall,A.C. (2010) A key role for membrane transporter NKCC1 in mediating chondrocyte volume increase in the mammalian growth plate. *J Bone Miner.Res.* **25**, 1594-1603.

22. Bush,P.G., Wokosin,D.L., & Hall,A.C. (2007) Two-versus one photon excitation laser scanning microscopy: critical importance of excitation wavelength. *Front Biosci.* **12**, 2646-2657.

23. Carlson,C.S., Loeser,R.F., Purser,C.B., Gardin,J.F., & Jerome,C.P. (1996) Osteoarthritis in cynomolgus macaques. III: Effects of age, gender, and subchondral bone thickness on the severity of disease. *J.Bone Miner.Res.* **11**, 1209-1217.

24. Chen,C.T., Bhargava,M., Lin,P.M., & Torzilli,P.A. (2003) Time, stress, and location dependent chondrocyte death and collagen damage in cyclically loaded articular cartilage. *J.Orthop.Res.* **21**, 888-898.

25. Chen,C.T., Burton-Wurster,N., Borden,C., Hueffer,K., Bloom,S.E., & Lust,G. (2001) Chondrocyte necrosis and apoptosis in impact damaged articular cartilage. *J.Orthop.Res.* **19**, 703-711.

26. Clark,J.M. (1990) The structure of vascular channels in the subchondral plate. *J.Anat.* **171**, 105-115.

27. Clark,J.M. & Simonian,P.T. (1997) Scanning electron microscopy of "fibrillated" and "malacic" human articular cartilage: technical considerations. *Microsc.Res.Tech.* **37**, 299-313.

28. D'Lima,D.D., Hashimoto,S., Chen,P.C., Colwell,C.W., Jr., & Lotz,M.K. (2001a) Human chondrocyte apoptosis in response to mechanical injury. *Osteoarthritis.Cartilage.* **9**, 712-719.

29. D'Lima,D.D., Hashimoto,S., Chen,P.C., Colwell,C.W., Jr., & Lotz,M.K. (2001b) Impact of mechanical trauma on matrix and cells. *Clin.Orthop.Relat Res.* S90-S99.

30. D'Lima,D.D., Hashimoto,S., Chen,P.C., Lotz,M.K., & Colwell,C.W., Jr. (2001c) Cartilage injury induces chondrocyte apoptosis. *J.Bone Joint Surg.Am.* **83-A** Suppl 2, 19-21.

31. D'Lima,D.D., Hashimoto,S., Chen,P.C., Lotz,M.K., & Colwell,C.W., Jr. (2001d) In vitro and in vivo models of cartilage injury. *J Bone Joint Surg Am* **83-A** Suppl 2, 22-24.

32. Dirschl,D.R., Marsh,J.L., Buckwalter,J.A., Gelberman,R., Olson,S.A., Brown,T.D., & Llinias,A. (2004) Articular fractures. *J Am Acad.Orthop.Surg* **12**, 416-423.

33. Eberhardt,A.W., Lewis,J.L., & Keer,L.M. (1991) Normal contact of elastic spheres with two elastic layers as a model of joint articulation. *J Biomech.Eng* **113**, 410-417.

34. Errington,R.J. & White,N.S. (1999) Measuring dynamic cell volume in situ by confocal microscopy. *Methods Mol.Biol.* **122**, 315-340.

35. Farndale,R.W., Buttle,D.J., & Barrett,A.J. (1986) Improved quantitation and discrimination of sulphated glycosaminoglycans by use of dimethylmethylene blue. *Biochim.Biophys.Acta* **883**, 173-177.

36. Farquhar,T., Xia,Y., Mann,K., Bertram,J., Burton-Wurster,N., Jelinski,L., & Lust,G. (1996) Swelling and fibronectin accumulation in articular cartilage explants after cyclical impact. *J.Orthop.Res.* **14**, 417-423.

37. Fell,H.B. (1969) Role of biological membranes in some skeletal reactions. *Ann.Rheum.Dis.* **28**, 213-227.

38. Fell,H.B. & Dingle,J.T. (1969) Endocytosis of sugars in embryonic skeletal tissues in organ culture. I. General introduction and histological effects. *J Cell Sci.* **4**, 89-103.

39. Fry,H.J. (1974) The interlocked stresses of articular cartilage. *Br.J.Plast.Surg.* **27**, 363-364.

40. Gilbert,S.J., Singhrao,S.K., Khan,I.M., Gonzalez,L.G., Thomson,B.M., Burdon,D., Duance,V.C., & Archer,C.W. (2009) Enhanced Tissue Integration During Cartilage Repair In Vitro Can Be Achieved by Inhibiting Chondrocyte Death at the Wound Edge. *Tissue Eng Part A* **15**, 1739-1749.

41. Goldberg,R.L., Spirito,S., Doughty,J.R., & Di Pasquale,G. (1993) Release of cell surface proteoglycan from chondrocytes by interleukin-1. *Agents Actions* **39**, C163-C165.

42. Goldring,M.B. (2000) The role of the chondrocyte in osteoarthritis. *Arthritis Rheum.* **43**, 1916-1926.

43. Goldring,M.B. (2006) Update on the biology of the chondrocyte and new approaches to treating cartilage diseases. *Best.Pract.Res.Clin.Rheumatol.* **20**, 1003-1025.

44. Grushko,G., Schneiderman,R., & Maroudas,A. (1989) Some biochemical and biophysical parameters for the study of the pathogenesis of osteoarthritis: a comparison between the processes of ageing and degeneration in human hip cartilage. *Connect.Tissue Res.* **19**, 149-176.

45. Grynpas,M.D., Alpert,B., Katz,I., Lieberman,I., & Pritzker,K.P. (1991) Subchondral bone in osteoarthritis. *Calcif.Tissue Int.* **49**, 20-26.

46. Guilak,F., Ratcliffe,A., & Mow,V.C. (1995) Chondrocyte deformation and local tissue strain in articular cartilage: a confocal microscopy study. *J.Orthop.Res.* **13**, 410-421.

47. Guilak,F., Zell,R.A., Erickson,G.R., Grande,D.A., Rubin,C.T., McLeod,K.J., & Donahue,H.J. (1999) Mechanically induced calcium waves in articular chondrocytes are inhibited by gadolinium and amiloride. *J Orthop.Res.* **17**, 421-429.

48. Gunal,I., Turgut,A., Acar,S., Tuc,A., Gokturk,E., & Karatosun,V. (2000) Effects of various irrigating solutions on articular cartilage. An experimental study in rabbits. *Bull.Hosp.Jt.Dis.* **59**, 73-75.

49. Hajek,P.C., Sartoris,D.J., Gylys-Morin,V., Haghighi,P., Engel,A., Kramer,F., Neumann,C.H., & Resnick,D. (1990) The effect of intra-articular gadolinium-DTPA on synovial membrane and cartilage. *Invest Radiol.* **25**, 179-183.

50. Hilal,G., Martel-Pelletier,J., Pelletier,J.P., Ranger,P., & Lajeunesse,D. (1998) Osteoblast-like cells from human subchondral osteoarthritic bone

demonstrate an altered phenotype in vitro: possible role in subchondral bone sclerosis. *Arthritis Rheum.* **41**, 891-899.

51. Hoffmann,E.K. & Dunham,P.B. (1995) Membrane mechanisms and intracellular signalling in cell volume regulation. *Int.Rev.Cytol.* **161**, 173-262.

52. Hughes,L.C., Archer,C.W., & Gwynn, I. (2005) The ultrastructure of mouse articular cartilage: collagen orientation and implications for tissue functionality. A polarised light and scanning electron microscope study and review. *Eur.Cell Mater.* **9**, 68-84.

53. Huntley,J.S., Bush,P.G., McBirnie,J.M., Simpson,A.H., & Hall,A.C. (2005a) Chondrocyte death associated with human femoral osteochondral harvest as performed for mosaicplasty. *J.Bone Joint Surg.Am.* **87**, 351-360.

54. Huntley,J.S., McBirnie,J.M., Simpson,A.H., & Hall,A.C. (2005b) Cutting-edge design to improve cell viability in osteochondral grafts. *Osteoarthritis.Cartilage.* **13**, 665-671.

55. Huntley,J.S., Simpson,A.H., & Hall,A.C. (2005c) Use of non-degenerate human osteochondral tissue and confocal laser scanning microscopy for the study of chondrocyte death at cartilage surgery. *Eur.Cell Mater.* **9**, 13-22.

56. Hunziker,E.B. (2002) Articular cartilage repair: basic science and clinical progress. A review of the current status and prospects. *Osteoarthritis.Cartilage.* **10**, 432-463.

57. Hunziker,E.B., Quinn,T.M., & Hauselmann,H.J. (2002) Quantitative structural organization of normal adult human articular cartilage. *Osteoarthritis.Cartilage.* **10**, 564-572.

58. Huser,C.A. & Davies,M.E. (2007) Calcium signaling leads to mitochondrial depolarization in impact-induced chondrocyte death in equine articular cartilage explants. *Arthritis Rheum.* **56**, 2322-2334.

59. Imhof,H., Breitenseher,M., Kainberger,F., Rand,T., & Trattnig,S. (1999) Importance of subchondral bone to articular cartilage in health and disease. *Top.Magn Reson.Imaging* **10**, 180-192.

60. Imhof,H., Sulzbacher,I., Grampp,S., Czerny,C., Youssefzadeh,S., & Kainberger,F. (2000) Subchondral bone and cartilage disease: a rediscovered functional unit. *Invest Radiol.* **35**, 581-588.

61. Jackson,D.W., Lalor,P.A., Aberman,H.M., & Simon,T.M. (2001) Spontaneous repair of full-thickness defects of articular cartilage in a goat model. A preliminary study. *J.Bone Joint Surg.Am.* **83-A**, 53-64.

62. Jadin,K.D., Wong,B.L., Bae,W.C., Li,K.W., Williamson,A.K., Schumacher,B.L., Price,J.H., & Sah,R.L. (2005) Depth-varying density and organization of chondrocytes in immature and mature bovine articular

cartilage assessed by 3d imaging and analysis. *J Histochem.Cytochem.* **53**, 1109-1119.

63. Jeffery,A.K., Blunn,G.W., Archer,C.W., & Bentley,G. (1991) Three-dimensional collagen architecture in bovine articular cartilage. *J.Bone Joint Surg.Br.* **73**, 795-801.

64. Jeffrey,J.E., Gregory,D.W., & Aspden,R.M. (1995) Matrix damage and chondrocyte viability following a single impact load on articular cartilage. *Arch.Biochem.Biophys.* **322**, 87-96.

65. Jochems,C.E., van der Valk,J.B., Stafleu,F.R., & Baumans,V. (2002) The use of fetal bovine serum: ethical or scientific problem? *Altern.Lab Anim* **30**, 219-227.

66. Jomha,N.M., Anoop,P.C., Elliott,J.A., Bagnall,K., & McGann,L.E. (2003) Validation and reproducibility of computerised cell-viability analysis of tissue slices. *BMC.Musculoskelet.Disord.* **4**, 5.

67. Jones,C.W., Smolinski,D., Keogh,A., Kirk,T.B., & Zheng,M.H. (2005) Confocal laser scanning microscopy in orthopaedic research. *Prog.Histochem.Cytochem.* **40**, 1-71.

68. Jones,K.H. & Senft,J.A. (1985) An improved method to determine cell viability by simultaneous staining with fluorescein diacetate-propidium iodide. *J Histochem.Cytochem.* **33**, 77-79.

69. Kerrigan,M.J., Hook,C.S., Qusous,A., & Hall,A.C. (2006) Regulatory volume increase (RVI) by in situ and isolated bovine articular chondrocytes. *J Cell Physiol* **209**, 481-492.

70. King,J., Bulstrode,C., & Revell,P. (1984) Irrigating fluid in arthroscopy. *Lancet* **1**, 159-160.

71. Knight,M.M., Roberts,S.R., Lee,D.A., & Bader,D.L. (2003) Live cell imaging using confocal microscopy induces intracellular calcium transients and cell death. *Am J Physiol Cell Physiol* **284**, C1083-C1089.

72. Korhonen,R.K., Wong,M., Arokoski,J., Lindgren,R., Helminen,H.J., Hunziker,E.B., & Jurvelin,J.S. (2002) Importance of the superficial tissue layer for the indentation stiffness of articular cartilage. *Med Eng Phys.* **24**, 99-108.

73. Krueger,J.A., Thisse,P., Ewers,B.J., Dvoracek-Driksna,D., Orth,M.W., & Haut,R.C. (2003) The extent and distribution of cell death and matrix damage in impacted chondral explants varies with the presence of underlying bone. *J.Biomech.Eng* **125**, 114-119.

74. Kuhn,K., D'Lima,D.D., Hashimoto,S., & Lotz,M. (2004) Cell death in cartilage. *Osteoarthritis.Cartilage.* **12**, 1-16.

75. Kwan,T.S., Lajeunesse,D., Pelletier,J.P., & Martel-Pelletier,J. (2010) Targeting subchondral bone for treating osteoarthritis: what is the evidence? *Best.Pract.Res.Clin.Rheumatol.* **24**, 51-70.

76. Lahm,A., Uhl,M., Erggelet,C., Haberstroh,J., & Mrosek,E. (2004) Articular cartilage degeneration after acute subchondral bone damage: an experimental study in dogs with histopathological grading. *Acta Orthop.Scand.* **75**, 762-767.

77. Lee,C.H., Cook,J.L., Mendelson,A., Moioli,E.K., Yao,H., & Mao,J.J. (2010) Regeneration of the articular surface of the rabbit synovial joint by cell homing: a proof of concept study. *Lancet* **376**, 440-448.

78. Lee,E.H. & Hui,J.H. (2006) The potential of stem cells in orthopaedic surgery. *J Bone Joint Surg Br* **88**, 841-851.

79. Levin,A., Burton-Wurster,N., Chen,C.T., & Lust,G. (2001) Intercellular signaling as a cause of cell death in cyclically impacted cartilage explants. *Osteoarthritis.Cartilage.* **9**, 702-711.

80. Lewis,J.L., Deloria,L.B., Oyen-Tiesma,M., Thompson,R.C., Jr., Ericson,M., & Oegema,T.R., Jr. (2003) Cell death after cartilage impact occurs around matrix cracks. *J.Orthop.Res.* **21**, 881-887.

81. Lin,G., Bjornsson,C.S., Smith,K.L., Abdul-Karim,M.A., Turner,J.N., Shain,W., & Roysam,B. (2005) Automated image analysis methods for 3-D quantification of the neurovascular unit from multichannel confocal microscope images. *Cytometry A* **66**, 9-23.

82. Loening,A.M., James,I.E., Levenston,M.E., Badger,A.M., Frank,E.H., Kurz,B., Nuttall,M.E., Hung,H.H., Blake,S.M., Grodzinsky,A.J., & Lark,M.W. (2000) Injurious mechanical compression of bovine articular cartilage induces chondrocyte apoptosis. *Arch.Biochem.Biophys.* **381**, 205-212.

83. Mainil-Varlet,P., Aigner,T., Brittberg,M., Bullough,P., Hollander,A., Hunziker,E., Kandel,R., Nehrer,S., Pritzker,K., Roberts,S., & Stauffer,E. (2003) Histological assessment of cartilage repair: a report by the Histology Endpoint Committee of the International Cartilage Repair Society (ICRS). *J.Bone Joint Surg.Am.* **85-A** Suppl 2, 45-57.

84. Malinin,T. & Ouellette,E.A. (2000) Articular cartilage nutrition is mediated by subchondral bone: a long-term autograft study in baboons. *Osteoarthritis.Cartilage.* **8**, 483-491.

85. Mankin,H.J. (1982) The response of articular cartilage to mechanical injury. *J Bone Joint Surg Am* **64**, 460-466.

86. Mansell,J.P. & Bailey,A.J. (1998) Abnormal cancellous bone collagen metabolism in osteoarthritis. *J.Clin.Invest* **101**, 1596-1603.

87. Mansell,J.P., Collins,C., & Bailey,A.J. (2007) Bone, not cartilage, should be the major focus in osteoarthritis. *Nat.Clin.Pract.Rheumatol.* **3**, 306-307.

88. Mansell,J.P., Tarlton,J.F., & Bailey,A.J. (1997) Biochemical evidence for altered subchondral bone collagen metabolism in osteoarthritis of the hip. *Br.J.Rheumatol.* **36**, 16-19.

89. Mansfield,K., Pucci,B., Adams,C.S., & Shapiro,I.M. (2003) Induction of apoptosis in skeletal tissues: phosphate-mediated chick chondrocyte apoptosis is calcium dependent. *Calcif.Tissue Int.* **73**, 161-172.

90. Maroudas, A. (1973) Physiochemical properties of articular cartilage. Adult Articular Cartilage. London, Pitman Medical, 131-170.

91. Maroudas,A. (1972) Physical chemistry and the structure of cartilage. *J Physiol* **223**, 21P-22P.

92. Maroudas,A., Bullough,P., Swanson,S.A., & Freeman,M.A. (1968) The permeability of articular cartilage. *J.Bone Joint Surg.Br.* **50**, 166-177.

93. Maroudas, A. & Kuettner K. (1990) Methods in Cartilage Research. London, Academic Press Limited, 211-219

94. Maroudas,A., Muir,H., & Wingham,J. (1969) The correlation of fixed negative charge with glycosaminoglycan content of human articular cartilage. *Biochim.Biophys.Acta* **177**, 492-500.

95. Maroudas,A. & Venn,M. (1977) Chemical composition and swelling of normal and osteoarthrotic femoral head cartilage. II. Swelling. *Ann.Rheum.Dis.* **36**, 399-406.

96. Maroudas,A., Ziv,I., Weisman,N., & Venn,M. (1985) Studies of hydration and swelling pressure in normal and osteoarthritic cartilage. *Biorheology* **22**, 159-169.

97. Maroudas,A.I. (1976) Balance between swelling pressure and collagen tension in normal and degenerate cartilage. *Nature* **260**, 808-809.

98. Mattson,M.P. (2006) Neuronal life-and-death signaling, apoptosis, and neurodegenerative disorders. *Antioxid.Redox.Signal.* **8**, 1997-2006.

99. Mattson,M.P. & Chan,S.L. (2003) Calcium orchestrates apoptosis. *Nat.Cell Biol.* **5**, 1041-1043.

100. McGann,L.E., Stevenson,M., Muldrew,K., & Schachar,N. (1988) Kinetics of osmotic water movement in chondrocytes isolated from articular cartilage and applications to cryopreservation. *J.Orthop.Res.* **6**, 109-115.

101. McGann,L.E., Yang,H.Y., & Walterson,M. (1988) Manifestations of cell damage after freezing and thawing. *Cryobiology* **25**, 178-185.

102. McKibbin, B. (1973) Nutrition. Adult Articular Cartilage. London, Pitman Medical, 277-286.

103. Milentijevic,D., Helfet,D.L., & Torzilli,P.A. (2003) Influence of stress magnitude on water loss and chondrocyte viability in impacted articular cartilage. *J.Biomech.Eng* **125**, 594-601.

104. Minsky M (1988) Memoir on inventing the confocal microscope. *Scanning* **10**, 128-138.

105. Mitchell,N. & Shepard,N. (1989) The deleterious effects of drying on articular cartilage. *J.Bone Joint Surg.Am.* **71**, 89-95.

106. Moldovan,F., Pelletier,J.P., Hambor,J., Cloutier,J.M., & Martel-Pelletier,J. (1997) Collagenase-3 (matrix metalloprotease 13) is preferentially localized in the deep layer of human arthritic cartilage in situ: in vitro mimicking effect by transforming growth factor beta. *Arthritis Rheum.* **40**, 1653-1661.

107. Mrosek,E.H., Lahm,A., Erggelet,C., Uhl,M., Kurz,H., Eissner,B., & Schagemann,J.C. (2006) Subchondral bone trauma causes cartilage matrix degeneration: an immunohistochemical analysis in a canine model. *Osteoarthritis.Cartilage* **14**, 171-178.

108. Murray,D.H., Bush,P.G., Brenkel,I.J., & Hall,A.C. (2010) Abnormal human chondrocyte morphology is related to increased levels of cell-associated IL-1beta and disruption to pericellular collagen type VI. *J Orthop.Res.* **28**, 1507-1514.

109. Neogi,T., Felson,D., Niu,J., Lynch,J., Nevitt,M., Guermazi,A., Roemer,F., Lewis,C.E., Wallace,B., & Zhang,Y. (2009a) Cartilage loss occurs in the same subregions as subchondral bone attrition: a within-knee subregion-matched approach from the Multicenter Osteoarthritis Study. *Arthritis Rheum.* **61**, 1539-1544.

110. Neogi,T., Nevitt,M.C., Niu,J., Sharma,L., Roemer,F., Guermazi,A., Lewis,C., Torner,J., Javaid,K., & Felson,D. (2009b) Subchondral bone attrition may be a reflection of compartment-specific mechanical load: The MOST Study. *Ann.Rheum.Dis.*

111. Pan,J., Zhou,X., Li,W., Novotny,J.E., Doty,S.B., & Wang,L. (2009) In situ measurement of transport between subchondral bone and articular cartilage. *J Orthop.Res.* **27**, 1347-1352.

112. Papacharalampous,X., Patsouris,E., Mundinger,A., Beck,A., Kouloulias,V., Primetis,E., Koureas,A., & Vlahos,L. (2005) The effect of contrast media on the synovial membrane. *Eur.J Radiol.* **55**, 426-430.

113. Pappin,D.J., Hojrup,P., & Bleasby,A.J. (1993) Rapid identification of proteins by peptide-mass fingerprinting. *Curr.Biol.* **3**, 327-332.

114. Parsch,D., Brummendorf,T.H., Richter,W., & Fellenberg,J. (2002) Replicative aging of human articular chondrocytes during ex vivo expansion. *Arthritis Rheum.* **46**, 2911-2916.

115. Pennock,A.T., Robertson,C.M., Wagner,F., Harwood,F.L., Bugbee,W.D., & Amiel,D. (2006a) Does subchondral bone affect the fate of osteochondral allografts during storage? *Am.J.Sports Med.* **34**, 586-591.

116. Pennock,A.T., Wagner,F., Robertson,C.M., Harwood,F.L., Bugbee,W.D., & Amiel,D. (2006b) Prolonged storage of osteochondral allografts: does the addition of fetal bovine serum improve chondrocyte viability? *J.Knee.Surg.* **19**, 265-272.

117. Pittenger,M.F., Mackay,A.M., Beck,S.C., Jaiswal,R.K., Douglas,R., Mosca,J.D., Moorman,M.A., Simonetti,D.W., Craig,S., & Marshak,D.R. (1999) Multilineage potential of adult human mesenchymal stem cells. *Science* **284**, 143-147.

118. Poole,C.A. (1997) Articular cartilage chondrons: form, function and failure. *J Anat.* **191**, 1-13.

119. Poole,C.A., Ayad,S., & Gilbert,R.T. (1992) Chondrons from articular cartilage. V. Immunohistochemical evaluation of type VI collagen organisation in isolated chondrons by light, confocal and electron microscopy. *J Cell Sci.* **103**, 1101-1110.

120. Poole,C.A., Brookes,N.H., & Clover,G.M. (2003) Confocal imaging of the human keratocyte network using the vital dye 5-chloromethylfluorescein diacetate. *Clin.Experiment.Ophthalmol.* **31**, 147-154.

121. Poole,C.A., Flint,M.H., & Beaumont,B.W. (1984) Morphological and functional interrelationships of articular cartilage matrices. *J Anat.* **138**, 113-138.

122. Prasadam,I., Friis,T., Shi,W., van Gennip,S., Crawford,R., & Xiao,Y. (2010) Osteoarthritic cartilage chondrocytes alter subchondral bone osteoblast differentiation via MAPK signalling pathway involving ERK1/2. *Bone* **46**, 226-235.

123. Quinn,T.M., Allen,R.G., Schalet,B.J., Perumbuli,P., & Hunziker,E.B. (2001) Matrix and cell injury due to sub-impact loading of adult bovine articular cartilage explants: effects of strain rate and peak stress. *J.Orthop.Res.* **19**, 242-249.

124. Radin,E.L., Paul,I.L., & Tolkoff,M.J. (1970) Subchondral bone changes in patients with early degenerative joint disease. *Arthritis Rheum.* **13**, 400-405.

125. Radin,E.L. & Rose,R.M. (1986) Role of subchondral bone in the initiation and progression of cartilage damage. *Clin.Orthop.Relat Res.* 34-40.

126. Raghunath,J., Rollo,J., Sales,K.M., Butler,P.E., & Seifalian,A.M. (2007) Biomaterials and scaffold design: key to tissue-engineering cartilage. *Biotechnol.Appl.Biochem.* **46**, 73-84.

127. Reagan,B.F., McInerny,V.K., Treadwell,B.V., Zarins,B., & Mankin,H.J. (1983) Irrigating solutions for arthroscopy. A metabolic study. *J.Bone Joint Surg.Am.* **65**, 629-631.

128. Reboul,P., Pelletier,J.P., Tardif,G., Benderdour,M., Ranger,P., Bottaro,D.P., & Martel-Pelletier,J. (2001) Hepatocyte growth factor induction of collagenase 3 production in human osteoarthritic cartilage: involvement of the stress-activated protein kinase/c-Jun N-terminal kinase pathway and a sensitive p38 mitogen-activated protein kinase inhibitor cascade. *Arthritis Rheum.* **44**, 73-84.

129. Redler,I., Mow,V.C., Zimny,M.L., & Mansell,J. (1975) The ultrastructure and biomechanical significance of the tidemark of articular cartilage. *Clin.Orthop.Relat Res.* 357-362.

130. Redman,S.N., Dowthwaite,G.P., Thomson,B.M., & Archer,C.W. (2004) The cellular responses of articular cartilage to sharp and blunt trauma. *Osteoarthritis.Cartilage.* **12**, 106-116.

131. Reindel,E.S., Ayroso,A.M., Chen,A.C., Chun,D.M., Schinagl,R.M., & Sah,R.L. (1995) Integrative repair of articular cartilage in vitro: adhesive strength of the interface region. *J Orthop.Res.* **13**, 751-760.

132. Richardson,S.M., Hoyland,J.A., Mobasheri,R., Csaki,C., Shakibaei,M., & Mobasheri,A. (2010) Mesenchymal stem cells in regenerative medicine: opportunities and challenges for articular cartilage and intervertebral disc tissue engineering. *J Cell Physiol* **222**, 23-32.

133. Rizzo,R., Grandolfo,M., Godeas,C., Jones,K.W., & Vittur,F. (1995) Calcium, sulfur, and zinc distribution in normal and arthritic articular equine cartilage: a synchrotron radiation-induced X-ray emission (SRIXE) study. *J Exp.Zool.* **273**, 82-86.

134. Roach,H.I., Shearer,J.R., & Archer,C. (1989) The choice of an experimental model. A guide for research workers. *J.Bone Joint Surg.Br.* **71**, 549-553.

135. Roberts,S.R., Knight,M.M., Lee,D.A., & Bader,D.L. (2001) Mechanical compression influences intracellular Ca2+ signaling in chondrocytes seeded in agarose constructs. *J Appl.Physiol* **90**, 1385-1391.

136. Rolauffs,B., Williams,J.M., Grodzinsky,A.J., Kuettner,K.E., & Cole,A.A. (2008) Distinct horizontal patterns in the spatial organization of superficial zone chondrocytes of human joints. *J Struct.Biol.* **162**, 335-344.

137. Sanchez,C., Deberg,M.A., Piccardi,N., Msika,P., Reginster,J.Y., & Henrotin,Y.E. (2005a) Osteoblasts from the sclerotic subchondral bone

downregulate aggrecan but upregulate metalloproteinases expression by chondrocytes. This effect is mimicked by interleukin-6, -1beta and oncostatin M pre-treated non-sclerotic osteoblasts. *Osteoarthritis.Cartilage* **13**, 979-987.

138. Sanchez,C., Deberg,M.A., Piccardi,N., Msika,P., Reginster,J.Y., & Henrotin,Y.E. (2005b) Subchondral bone osteoblasts induce phenotypic changes in human osteoarthritic chondrocytes. *Osteoarthritis.Cartilage* **13**, 988-997.

139. Schumacher,B.L., Block,J.A., Schmid,T.M., Aydelotte,M.B., & Kuettner,K.E. (1994) A novel proteoglycan synthesized and secreted by chondrocytes of the superficial zone of articular cartilage. *Arch.Biochem.Biophys.* **311**, 144-152.

140. Schumacher,B.L., Su,J.L., Lindley,K.M., Kuettner,K.E., & Cole,A.A. (2002) Horizontally oriented clusters of multiple chondrons in the superficial zone of ankle, but not knee articular cartilage. *Anat.Rec.* **266**, 241-248.

141. Setton,L.A., Tohyama,H., & Mow,V.C. (1998) Swelling and curling behaviors of articular cartilage. *J Biomech.Eng* **120**, 355-361.

142. Shapiro,F., Koide,S., & Glimcher,M.J. (1993) Cell origin and differentiation in the repair of full-thickness defects of articular cartilage. *J.Bone Joint Surg.Am.* **75**, 532-553.

143. Simpkin,V.L., Murray,D.H., Hall,A.P., & Hall,A.C. (2007) Bicarbonate-dependent pH(i) regulation by chondrocytes within the superficial zone of bovine articular cartilage. *J Cell Physiol* **212**, 600-609.

144. Squires,G.R., Okouneff,S., Ionescu,M., & Poole,A.R. (2003) The pathobiology of focal lesion development in aging human articular cartilage and molecular matrix changes characteristic of osteoarthritis. *Arthritis Rheum.* **48**, 1261-1270.

145. Stockwell & Meachim 1973, R.A. & Meachim, G. (1973) The Chondrocytes. Adult Articular Cartilage. London, Pitman Medical, 51-99.

146. Temenoff,J.S. & Mikos,A.G. (2000) Review: tissue engineering for regeneration of articular cartilage. *Biomaterials* **21**, 431-440.

147. Tew,S.R., Kwan,A.P., Hann,A., Thomson,B.M., & Archer,C.W. (2000) The reactions of articular cartilage to experimental wounding: role of apoptosis. *Arthritis Rheum.* **43**, 215-225.

148. Torzilli,P.A., Grigiene,R., Borrelli,J., Jr., & Helfet,D.L. (1999) Effect of impact load on articular cartilage: cell metabolism and viability, and matrix water content. *J.Biomech.Eng* **121**, 433-441.

149. Uozumi,H., Sugita,T., Aizawa,T., Takahashi,A., Ohnuma,M., & Itoi,E. (2009) Histologic findings and possible causes of osteochondritis dissecans of the knee. *Am J Sports Med* **37**, 2003-2008.

150. Urban,J.P. (1994) The chondrocyte: a cell under pressure. *Br.J.Rheumatol.* **33**, 901-908.

151. Urban,J.P., Hall,A.C., & Gehl,K.A. (1993) Regulation of matrix synthesis rates by the ionic and osmotic environment of articular chondrocytes. *J.Cell Physiol* **154**, 262-270.

152. van Susante,J.L., Buma,P., van Beuningen,H.M., van den Berg,W.B., & Veth,R.P. (2000) Responsiveness of bovine chondrocytes to growth factors in medium with different serum concentrations. *J.Orthop.Res.* **18**, 68-77.

153. Westacott,C. (2002) Interactions between subchondral bone and cartilage in OA. Cells from osteoarthritic bone can alter cartilage metabolism. *J.Musculoskelet.Neuronal.Interact.* **2**, 507-509.

154. Westacott,C.I., Webb,G.R., Warnock,M.G., Sims,J.V., & Elson,C.J. (1997) Alteration of cartilage metabolism by cells from osteoarthritic bone. *Arthritis Rheum.* **40**, 1282-1291.

155. Wilkins,R.J. & Hall,A.C. (1992) Measurement of intracellular pH in isolated bovine articular chondrocytes. *Exp.Physiol* **77**, 521-524.

156. Williams,S.K., Amiel,D., Ball,S.T., Allen,R.T., Wong,V.W., Chen,A.C., Sah,R.L., & Bugbee,W.D. (2003) Prolonged storage effects on the articular cartilage of fresh human osteochondral allografts. *J.Bone Joint Surg.Am.* **85-A**, 2111-2120.

157. Wong,M., Wuethrich,P., Eggli,P., & Hunziker,E. (1996) Zone-specific cell biosynthetic activity in mature bovine articular cartilage: a new method using confocal microscopic stereology and quantitative autoradiography. *J.Orthop.Res.* **14**, 424-432.

158. Yang,C.Y., Cheng,S.C., & Shen,C.L. (1993) Effect of irrigation fluids on the articular cartilage: a scanning electron microscope study. *Arthroscopy* **9**, 425-430.

159. Zahir,A. & Freeman,A.R. (1972) Cartilage changes following a single episode of infarction of the capital femoral epiphysis in the dog. *J.Bone Joint Surg.Am.* **54**, 125-136.

160. Zhu,Y., Demilie,P., Davoine,P., Cartage,T., & Delplancke-Ogletree,M. (2005) Influence of calcium ions on the crystallization of sodium bicarbonate. *Journal of Crystal Growth* **275**, 1333-1339.

## APPENDIX

I. List of published papers, abstracts, presentations and prizes related to this work

II. Reprints of five published papers based on this work

III. Letter from the Local Research Ethics Committee permitting the use of human tissue related to this work

## APPENDIX I

## List of published papers, abstracts, presentations and prizes related to this work

### Published papers

1. Amin AK, Huntley JS, Bush PG, Simpson AHRW, Hall AC. Osmolarity influences chondrocyte death in wounded articular cartilage. *J Bone Joint Surg (Am).* 2008; 90:1531-1542
   **(Based on Chapter 3 of this thesis)**

2. Amin AK, Huntley JS, Bush PG, Simpson AHRW, Hall AC. Chondrocyte death in mechanically injured articular cartilage – the influence of extracellular calcium. *J Orthop Res.* 2008; 27:778-784
   **(Based on Chapter 4 of this thesis)**

3. Amin AK, Huntley JS, Simpson AHRW, Hall AC. Increasing the osmolarity of joint irrigation solutions may avoid injury to cartilage: A pilot study. *Clin Orthop Relat Res.* 2010; 468:875-884
   **(Based on Chapter 5 of this thesis)**

4. Amin AK, Huntley JS, Patton JT, Brenkel IJ, Simpson AHRW, Hall AC. Hyperosmolarity protects chondrocytes from mechanical injury in human articular cartilage. An experimental report. *J Bone Joint Surg(Br).* 2011; 93:277-284
   **(Based on Chapter 6 of this thesis)**

5. Amin AK, Huntley JS, Simpson AHRW, Hall AC. Chondrocyte survival in articular cartilage explants – the influence of subchondral bone in a bovine model. *J Bone and Joint Surg(Br).* 2009; 91:691-699
   **(Based on Chapter 7 of this thesis)**

   NB: A manuscript based on the results presented in Chapter 8 of this thesis is currently in preparation

### Published abstracts

1. Amin AK, Huntley JS, Simpson AHRW, Hall AC. Chondroprotective strategies: increasing the osmolarity of joint irrigating solutions. *J Bone Joint Surg(Br).* 2010; 92-B:Supp 1, 60

2. Amin AK, Huntley JS, Simpson AHRW, Hall AC. Chondrocyte survival in articular cartilage explants – the influence of subchondral bone. *J Bone Joint Surg(Br).* 2010; 92-B:Supp 1, 56

3. Amin AK, Huntley JS, Bush PG, Simpson AHRW, Hall AC. Chondrocyte death in mechanically injured articular cartilage – the influence of extracellular calcium. *Proceedings: 2nd Joint meeting of the Bone Research Society and British Orthopaedic Research Society, Manchester, UK, 23-25 June, 2008*

4. Amin AK, Huntley JS, Simpson AHRW, Hall AC. Medium osmolarity influences chondrocyte death in injured articular cartilage: implications for the design of joint irrigation solutions. *Proceedings: Scottish Orthopaedic Meeting, June 13, 2008 (published in the Scottish Medical Journal)*

5. Amin AK, Huntley JS, Simpson AHRW, Hall AC. The influence of subchondral bone on chondrocyte survival in bovine cartilage. *Osteoarthritis and Cartilage* 2007; 15:Supp C 108-109

6. Amin AK, Huntley JS, Bush PG, Simpson AHRW, Hall AC. Osmolarity influences chondrocyte death in wounded articular cartilage. *Osteoarthritis and Cartilage* 2007; 15:Supp C 127-128

## Oral Presentations

1. The influence of subchondral bone on chondrocyte survival in human and bovine articular cartilage explants. Scottish Orthopaedic Meeting 12 June, Stirling, 2009

2. Chondrocyte survival in articular cartilage explants – the influence of subchondral bone. 2nd Joint meeting of the Bone Research Society and British Orthopaedic Research Society, Manchester, UK, 23-25 June, 2008

3. Chondroprotective strategies: increasing the osmolarity of joint irrigating solutions. 2nd Joint meeting of the Bone Research Society and British Orthopaedic Research Society, Manchester, UK, 23-25 June, 2008

4. Medium osmolarity influences chondrocyte death in injured articular cartilage: implications for the design of joint irrigation solutions. Roy Petrie Memorial meeting, Fife Acute Hospitals NHS Trust, April 11 2008

5. The effect of medium osmolarity on in situ chondrocyte death within wounded articular cartilage. 7th World Congress of the International Cartilage Repair Society, Warsaw, Poland, Sept 29-Oct 2, 2007

## Poster presentations

1. Amin AK, Huntley JS, Simpson AHRW, Hall AC. Bone-cartilage interactions: the survival of human articular chondrocytes is influenced by subchondral bone. 55th Annual meeting of the Orthopaedic Research Society, Las Vegas, USA, February 22-25, 2009

2. Amin AK, Huntley JS, Simpson AHRW, Hall AC. Chondroprotective strategies: increasing the osmolarity of joint irrigating solutions. 54th Annual meeting of the Orthopaedic Research Society, San Francisco, USA, March 2-5, 2008

3. Amin AK, Huntley JS, Bush PG, Simpson AHRW, Hall AC. Chondrocyte death in mechanically injured articular cartilage – the influence of extracellular calcium. 2nd Joint meeting of the Bone Research Society and British Orthopaedic Research Society, Manchester, UK, 23-25 June, 2008

4. Amin AK, Huntley JS, Simpson AHRW, Hall AC. Medium osmolarity influences chondrocyte death in injured articular cartilage: implications for the design of joint irrigation solutions. Scottish Orthopaedic Meeting, June 13, 2008

5. Amin AK, Huntley JS, Bush PG, Simpson ARWH, Hall AC. Osmolarity influences chondrocyte death in wounded articular cartilage. OARSI World Congress on Osteoarthritis, Florida, USA December 6-9, 2007

6. Amin AK, Huntley JS, Simpson ARWH, Hall AC. The influence of subchondral bone on chondrocyte survival in bovine cartilage. OARSI World Congress on Osteoarthritis, Florida, USA December 6-9, 2007

## Prizes

1. Best Oral Presentation by a Clinical Scientist, British Orthopaedic Research Society (BORS), Manchester, UK
   Chondrocyte survival in articular cartilage explants – the influence of subchondral bone. 2nd Joint meeting of the Bone Research Society and British Orthopaedic Research Society, Manchester, UK, 23-25 June, 2008

2. Best Poster, Scottish Orthopaedic Meeting, Stirling, Scotland.
   Medium osmolarity influences chondrocyte death in injured articular cartilage: implications for the design of joint irrigation solutions. Scottish Orthopaedic Meeting, June 13, 2008

## APPENDIX II

**Reprints of five published papers based on this work**

## APPENDIX III

## Letter from the Local Research Ethics Committee permitting the use of human tissue related to this work